THE MANY FACES OF TWO CLOSELY RELATED TAUOPATHIES: PROGRESSIVE SUPRANUCLEAR PALSY AND CORTICOBASAL DEGENERATION

by

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This thesis would not have been possible without the continuous and unreserved support of my wife, Marta. Finally, I would like to dedicate this thesis to the memory of my brother, Miklós and my parents, Berta and József.

Submission declaration

I declare that the submitted material as a whole is not substantially the same as published or unpublished material that I have previously submitted, or am currently submitting, for a degree, diploma, or similar qualification at any university or similar institution. Parts of the material published in papers 2, 3 and 4 were included into the theses of two of my PhD students, as indicated in the document outlining my contribution to the published work on pages 6 and 7.

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Publications submitted for consideration for the degree of Doctor of Philosophy by Published Work with statements of the candidate's contribution to the published work

Papers with Statement of Contribution	Authorship
Paper 1 **The nucleus raphe interpositus in the Steele-Richardson-Olszewski syndrome (progressive supranuclear palsy).** ***Brain 1996; 119, 1137-1143*** Tamas Revesz conceived the idea of this research. Neuropathological review by SE Daniel and T Revesz, tissue sections were stained by Ms Sangha (pathology technician). T Revesz designed the methods and carried out the morphometry, evaluated the data, carried out the statistical tests and wrote the first draft of the paper. SE Daniel contributed to the final version of the paper.	**Revesz T*** Sangha H Daniel SE
Paper 2 **Pathological, clinical and genetic heterogeneity in progressive supranuclear palsy.** ***Brain 2002; 125:969-975*** The idea of the project was conceived by T Revesz, SE Daniel, BH Anderton. Neuropathological review of the cases was carried out by T Revesz and SE Daniel. Tau protein extraction and western blot analysis were carried out by T Revesz, G Gibb, D Hanger (T Revesz was supervised by G Gibb and BH Anderton). Data evaluation by D Hanger, Dr Gibb, T Revesz with advice from BH Anderton. Genetic analysis: HR Morris under the supervision of NW Wood. C Strand and T Lashley carried tau immunohistochemistry. The manuscript was written by HR Morris, G Gibb and T Revesz with input from the other co-authors. Addendum: Parts of this work is included in HR Morris's PhD thesis	Morris HR Gibb G Katzenschlager Wood NW Hanger DP Strand C Lashley T Daniel SE Lees AJ Anderton BH **Revesz T***
Paper 3 **Pathological tau burden and distribution distinguishes progressive supranuclear palsy-parkinsonism from Richardson's syndrome.** ***Brain 2007; 130:1566-1576*** The idea of this neuropathological project was conceived by DR Williams, T Revesz, AJ Lees. Neuropathological review of the cases was carried out by T Revesz and JL Holton. The morphometry was designed by DR Williams and T Revesz as co-supervisor of DR Williams's PhD project. DR Williams carried out the data acquisition. Quality control: T Revesz. Data evaluation by statistical methods by DR Williams, discussing the results with T Revesz. The first draft of the manuscript was written by DR Williams. T Revesz, J Holton and AJ Lees helped with the final, published version of the manuscript.	Williams DR Holton JL Strand C Pittman A de Silva R Lees AJ **Revesz T***

Paper 4 **Characteristics of progressive supranuclear palsy presenting with corticobasal syndrome: a cortical variant** ***Neuropathol Appl Neurobiol 2014; 40:149–163*** This neuropathological project was conceived and the methodology designed by T Revesz as the neuropathologist co-supervisor of H Ling's PhD project. Review of clinical aspects of the cases: AJ Lees, H Ling. Neuropathological review: T Revesz. Morphometry and data analysis, including statistics: H Ling. Quality control: T Revesz. Western blotting for tau: R de Silva. The first draft of the manuscript was prepared by H Ling. T Revesz, AJ Lees and JL Holton contributed to the final, accepted manuscript.	Ling H De Silva R Massey LA Courtney R Hondhamuni G Bajaj N Lowe J Holton JL Lees AJ **Revesz T***
Paper 5 **Astrogliopathy predominates the earliest stage of corticobasal degeneration pathology.** ***Brain 2016; 139; 3237–3252*** This is part of a large, still ongoing project supported by a grant (PI: T Revesz). Design of project: T Revesz. Neuropathological review: T Revesz, JL Holton and H Ling. H Ling was responsible for carrying out the morphometry and data analysis including statistics under the supervision of T Revesz. Regular quality control: T Revesz. Tau immunohistochemistry, digitising stained slides, arranging automated image analysis: K Davey. Genetics: KY Mok under the supervision of J Hardy. GG Kovacs and Vonsattel contributed by providing one case each. HR Morris and TT Warner gave advice on clinical aspects. With advice/help from T Revesz, H Ling prepared the first draft and the final form of the manuscript.	Ling H Kovacs GG Vonsattel JPG Davey K Mok KY Hardy J Morris HR Warner TT Holton JL **Revesz T***
***Denotes corresponding and senior authorship** *Copies of these statements, signed by the first authors and other authors who played an important role in the study, are attached in Appendix A*	

1. SUMMARY OF THESIS

The five studies forming this thesis deal with two closely related neurodegenerative diseases, progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD), both of which are characterised by abundant tau-positive neuronal and glial filamentous inclusions and by shared genetic risk factors[19, 70]. Four studies focus on demonstrating neuropathological, biochemical and genetic differences between the classical/typical form and atypical PSP while the fifth study discusses issues relevant for understanding disease progression in CBD.

Paper 1: In this study[64] we used a stereological tool (optical disector) for the investigation of the nucleus raphe interpositus containing the omnipause neurons, whose integrity is essential for normal saccadic eye movements. We found that the loss of the omnipause neurons and the degree of neurofibrillary degeneration in this nucleus is significantly greater in typical PSP than in the atypical variant, defined in this study by the absence of supranuclear gaze palsy (SGP), which is the classical eye movement sign in PSP[64].

Paper 2: In the second study[55], in addition to neuropathological assessment of the cases, we carried out protein analysis of PSP-tau and investigated the tau gene (*MAPT*) haplotypes in clinically typical and atypical PSP cases. The atypical cases were clinically more heterogeneous than those used in **paper 1**; a minority of the cases was lacking SGP and some showed a Parkinson's disease-like clinical presentation. We found that the characteristic 'doublet' western blot pattern of PSP-tau, indicating that it primarily consists of 4-repeat tau (4R-tau) isoforms, and the PSP genetic susceptibility H1 *MAPT* haplotype are strongly associated with typical PSP. A deviation from this was recognised in atypical PSP in that the H1 *MAPT* haplotype was less frequent and that the 3-repeat tau (3R-tau) isoforms made a significant contribution to PSP-tau[55]**.**

Paper 3: Two seminal studies from the Queen Square Brain Bank for Neurological Disorders, UCL Institute of Neurology (QSBB) provided a conceptual framework for reliably separating clinically classical/typical PSP (named Richardson's syndrome - PSP-RS) from two atypical variants, PSP-parkinsonism (PSP-P)[77] and PSP-pure akinesia with gait freezing (PSP-PAGF)[79]. Using these clinical categories, our third study[78] employing unbiased morphometry, showed marked differences in the cerebral tau-load between PSP-RS and

each of the two variants, PSP-P and PSP-PAGF in that these two atypical variants were associated with less severe and anatomically more restricted tau-burden than the clinically typical cases. Furthermore we could also demonstrate the pattern of the topographical expansion of the disease that is associated with an increase in the severity of the tau-burden[78].

Paper 4: Using unbiased quantitative neuropathological methods for establishing the cerebral tau-load, supplemented by western blot analysis of PSP-tau and investigation of the *MAPT* haplotypes, we also studied the clinically well-characterised PSP-corticobasal syndrome atypical variant (PSP-CBS)[46]. In this study, we could confirm findings of previous studies indicating that, compared with typical PSP, there is an increased cortical tau-burden in PSP-CBS. Furthermore, our study also demonstrated that, parallel with the increased cortical tau-burden, the basal ganglia tau-load is decreased in PSP-CBS, which explains why the total cerebral tau-burden (tau-load in all areas investigated) in PSP-CBS is similar to that can be found in PSP-RS. These findings also indicate that in PSP-CBS there is a marked shift of the tau-burden from the basal ganglia to neocortical areas[46].

Paper 5: As the patterns of progression of the tau pathology are poorly understood in PSP or CBD, in this study[47] we investigated a group of 'incidental' (preclinical, clinically asymptomatic) CBD cases with 'early' pathological changes by using tau immunohistochemistry and unbiased quantitative neuropathological methods. Genetic analysis of the tau (*MAPT*) gene was also carried out. We found no mutation in the *MAPT* gene in any of the 'incidental' cases, one of which had the H1/H2 *MAPT* haplotype while the remainder had the H1/H1 haplotype. The overall severity of the tau pathology was found to be significantly less and anatomically more restricted in 'incidental' CBD than in control CBD cases with 'end-stage' pathology. Furthermore, we also found that the earliest disease stages are dominated by astroglial tau pathology (astrocytic plaques). Analysis of the distribution of the tau pathology also indicates that striatal connections to the dorsolateral prefrontal cortex and basal ganglia circuitry are the earliest neural network connections that are affected by CBD tau pathology.

The findings of the first four studies underpinned the validity of the emerging concept of clinically diverse PSP variants as we could identify relevant differences in the severity and distribution of the tau pathology between typical PSP and the atypical variants. These findings also imply that as in Alzheimer's disease, the severity and topographical distribution of the tau pathology determine the clinical syndrome in both typical PSP and the atypical variants. Furthermore, in the atypical cases the H1/H1 tau haplotype was less frequent and a contribution by 3R-tau to PSP-tau was more common than in typical PSP.

Our fifth study identified for the first time that in CBD the astrocytic tau pathology is prominent in the earliest stages of the disease and that the earliest neuropathological changes occur in the basal ganglia and the prefrontal cortex. This latter observation has important implications on our understanding the topographical progression of the tau pathology in this condition.

2. LIST OF ABBREVIATIONS AND ACRONYMS

3R-tau	= 3-repeat tau
4R-tau	= 4-repeat tau
3R-tauopathy	= 3-repeat tauopathy
4R-tauopathy	= 4-repeat tauopathy
ALS/PD complex	= amyotrophic lateral sclerosis/parkinsonism complex
Aβ	= amyloid-β
CAA	= cerebral amyloid angiopathy
CB	= coiled body
CBD	= corticobasal degeneration
CBD-bvFTD	= CBD-behavioural variant frontotemporal dementia
CBD-CBS	= CBD-corticobasal syndrome
CBD-PPA	= CBD-primary progressive aphasia
CBD-PSPS	= CBD-progressive supranuclear palsy syndrome
CBD-RS	= CBD-Richardson's syndrome
CBS	= corticobasal syndrome
DNA	= deoxyribonucleic acid
EM	= electron microscopy
FTDP-17*MAPT*	= frontotemporal dementia and parkinsonism linked to chromosome 17, due to mutations in the *MAPT* gene
MAPT gene	= microtubule-associated protein tau gene
mRNA	= messenger ribonucleic acid
NFT	= neurofibrillary tangle
NINDS	= National Institute of Neurological Disorders and Stroke
PHF	= paired helical filament
PreT	= pretangle
PSP	= progressive supranuclear palsy
PSP-AOS	= PSP-apraxia of speech
PSP-C	= PSP-cerebellar
PSP-CBS	= PSP-corticobasal syndrome
PSP-FTD	= PSP-frontotemporal dementia
PSP-P	= PSP-parkinsonism
PSP-PAGF	= PPS-pure akinesia with gait freezing
PSP-PPA	= PSP-primary progressive aphasia
PSP-RS	= PSP-Richardson's syndrome
QSBB	= Queen Square Brain Bank for Neurological Disorders
SD	= standard deviation
SDS-PAGE	= sodium dodecyl sulphate–polyacrylamide
SEM	= standard error of the mean
SF	= straight filament
SGP	= supranuclear gaze palsy
Th	= neuropil thread
UCL	= University College London

3. BACKGROUND

3.1 Clinical definition of progressive supranuclear palsy and corticobasal degeneration

Progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD) are clinically and pathologically heterogeneous diseases which, based on their clinical presentations, are classified as atypical Parkinsonian disorders. PSP and CBD are closely-related conditions as they share genetic risk factors and biochemical characteristics of the microtubule-associated protein tau, which in both diseases forms abundant filamentous inclusions in both neurones and glial cells[19, 62]. Both PSP and CBD were systematically described only in the 1960s, although it is likely that isolated cases had been reported earlier[61, 69, 70].

3.1.1 Progressive supranuclear palsy

PSP is the most common atypical parkinsonian disorder[62]. In a large pathologically proven cohort from the QSBB, 63% of 103 cases, collected between 1988 and 2002 were male. The mean age at onset in this cohort was 66.4 years (SD: 12), the mean age at death 73.5 years (SD: 7.5) with a mean disease duration of 7.0 years (SD: 3.7)[77]. Using passive referral evaluation, the prevalence of classical PSP has been reported to be 0.1/100,000 in the UK while with active case ascertainment method, the crude and the age-adjusted prevalence was estimated to be 3.1/100,000 and 2.4/100,000, respectively[57].

The classical clinical syndrome of PSP includes postural instability, parkinsonism, ophthalmoplegia, which primarily affects downward gaze, pseudobulbar palsy, dysarthria, dystonic rigidity of the neck as well as upper trunk and mild dementia[69, 70]. The clinical signs reflect pathological involvement of numerous subcortical and brainstem structures such as those of the extrapyramidal motor systems or responsible for the supranuclear organisation of eye movements (see below)[19, 62].

In 1990s clinical diagnostic criteria were developed, which showed good specificity for cases with classical clinical presentation, but relatively low sensitivity[49, 50, 76]. These have been recently updated and modified under the aegis of the Movement Disorder Society[35]. Based on clinical diagnostic probability, categories of ‘possible’, ‘probable’ and ‘definite’ PSP were proposed. It is of note that for ‘definite’ PSP diagnosis histological confirmation of PSP is required[49, 50].

3.1.2 Corticobasal degeneration

CBD was first described clinically and neuropathologically under the term 'corticodentatonigral degeneration with neuronal achromasia'[61], which was later replaced by the term 'corticobasal degeneration' (CBD)[22]. As, in addition to CBD, a number of other conditions may also underlie the classical clinical presentation, the term corticobasal syndrome (CBS) is more appropriate for clinical diagnosis[45, 48].

CBD is a sporadic condition affecting both sexes equally. The calculated estimated incidence of CBD is thought to be less than 1/100,000 per year[19]. The average age at disease onset is ~60 years and the disease duration is between 6–10 years[62].

The classical clinical presentation (CBS) is characteristically asymmetrical with clumsiness, stiffness or myoclonic jerks of a limb. Dystonic rigidity, difficulty of walking, akinesia and cortical sensory signs are also features and some patients show 'alien limb' sign.

Consensus clinical diagnostic criteria have been established and, in addition to CBS (CBD-CBS), CBD pathology has been shown to underlie several other clinical syndromes. These include a PSP-like syndrome/Richardson's syndrome (CBD-PSPS/CBD-RS), behavioural variant of frontotemporal dementia (CBD-bvFTD), non-fluent/agrammatic variant of primary progressive aphasia (CBD-PPA) and rarely a presentation with cerebellar ataxia (CBD-OPCA)[4, 43-45, 48], underpinning the notion that CBD is a disease of both movement and cognition[62, 63].

3.2 Neuropathology

3.2.1 Progressive supranuclear palsy

In cases with classical clinical presentation, inspection of the brain reveals dilatation of the 3rd and 4th ventricles, severe atrophy of the subthalamus, tegmentum of the midbrain and pons, marked pallor of the substantia nigra, reduction in size and discolouration of the globus pallidus and, in most cases, severe atrophy of the superior cerebellar peduncle due to loss of neurons in the cerebellar dentate nucleus. Mild frontal atrophy may be a feature.

Microscopic investigation confirms neuronal cell loss and astrogliosis in structures showing macroscopic abnormality, including the subthalamic nucleus, globus pallidus, substantia nigra, in which both the pars compacta and pars reticulata are affected by severe neuronal loss, other brainstem nuclei and the cerebellar dentate nucleus[15, 19, 59, 62, 70].

Neurofibrillary tangles (NFTs), some with features of globose tangles (**Figure 1D**), can be recognised on the haematoxylin and eosin preparations. Tau immunohistochemistry reveals severe accumulation of disease-associated, hyperphosphorylated tau in both neurons as NFTs as well as pretangles (PreT) and glial cells in the basal ganglia, brainstem structures, including supranuclear gaze centres, cerebellar nuclei, spinal cord and also cerebral cortex (**Figure 1A, 1E)**[62]. Tau deposition in astrocytes gives rise to tufted astrocytes, which are readily seen in the frontal cortex including motor cortex, and striatum (**Figure 1A, 1B**). The stellate-shaped tufted astrocytes with accumulation of filamentous tau in the cytoplasm and proximal astrocytic processes, are pathological hallmarks of PSP and are required for the neuropathological diagnosis[19, 62]. Tau deposition in oligodendroglia gives rise to coiled bodies (CBs), which can be numerous among others in the internal capsule, cerebral peduncles and cerebellar white matter[19, 62] (**Figure 1C**). Tau-positive neuropil threads (Ths) are also widespread.

Electron microscopic examination of the tau filaments demonstrates ~15nm wide straight filaments.

Pathological criteria for the diagnosis of PSP were established and validated under the auspices of the NINDS[49, 51] in the 1990s, although these have become outdated and an update is required.

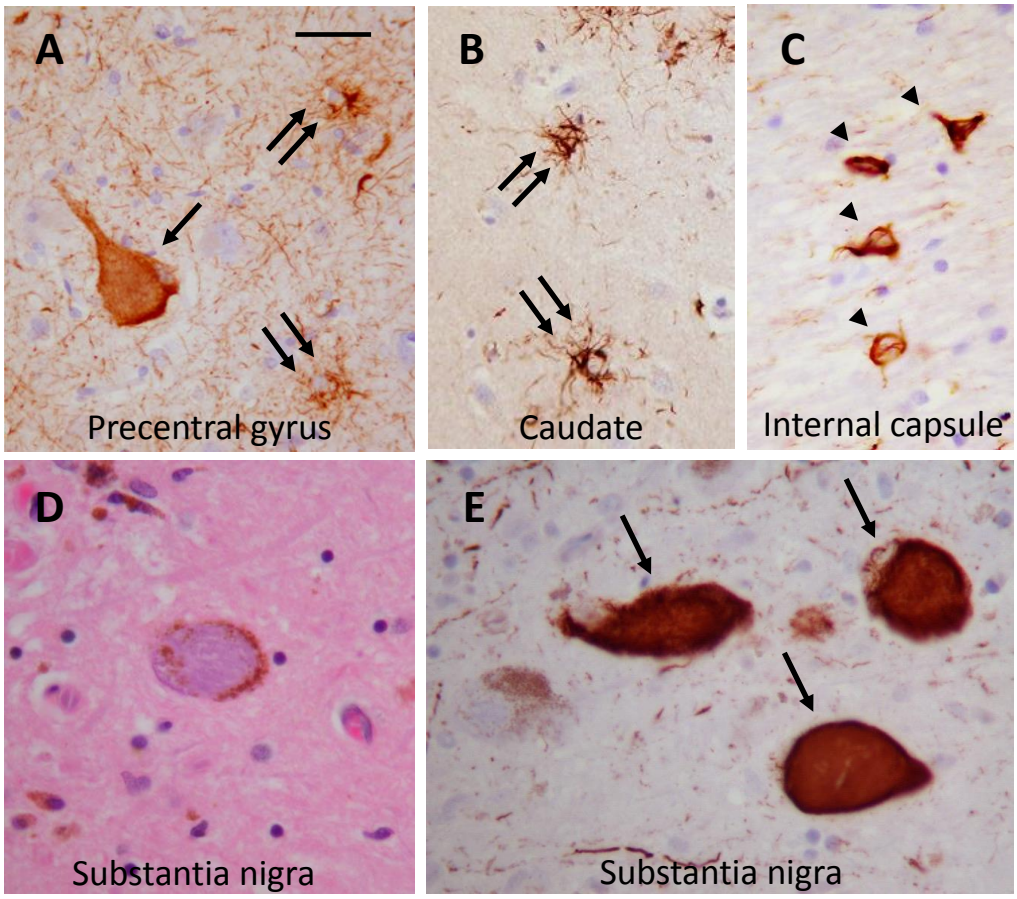

Figure 1 Main microscopic findings in progressive supranuclear palsy
Tau-positive neurofibrillary tangles (arrows) **(A**, **E),** tufted astrocytes (double arrows) (**A**, **B**) and coiled bodies (arrowheads) (**C**) are characteristic in progressive supranuclear palsy. Panel **D** shows a globose neurofibrillary tangle in a neuron of the substantia nigra. Panels **A**,**B**,**C** and **E** tau immunohistochemistry (AT8 antibody), panel **D** haematoxylin and eosin stain. Scale bar on panel **A** represents 22.5μm on panels **A** **C**, **D** and **E** and 90μm on panel **B**.

3.2.1 Corticobasal degeneration

Gross inspection of the brain reveals cortical atrophy of the posterior frontal and parietal cortices, which may be asymmetrical and may show a parasagittal distribution in cases presenting with CBS. The atrophy may be more apparent in the anterior frontal and temporal regions in CBD-bvFTD or CBD-PPA[19]. There is variable dilatation of the ventricular system; the cerebral hemispheric white matter, including the corpus callosum may be reduced in bulk. Atrophy of the subthalamic nucleus, prominent in PSP, is minimal or absent in CBD. The pallor of the substantia nigra and locus coeruleus is severe in cases with advanced disease[19, 62].

In CBD microscopy reveals marked neuronal loss with astrocytosis in severely affected cerebral cortices where vacuolation of the neuropil in the upper cortical laminae is seen. Swollen (ballooned or achromatic) cortical neurons, most frequent in deeper cortical layers, can be observed, although their presence is not required for the diagnosis as they may be absent[18]. The subcortical grey nuclei show variable neuronal loss, which is mostly severe in the substantia nigra. Degeneration of the corticospinal tracts may occur due to degeneration of the motor cortex and there is myelin pallor in the cerebral white matter.

Validated neuropathological diagnostic criteria of CBD emphasise the importance of tau-positive Ths in grey and white matter of the cerebral cortex (**Figure 2C, 2D**), basal ganglia and rostral brainstem (**Figure 2A-E**). Tau-positive neuronal (NFTs and PreTs) and glial filamentous inclusions are also widespread. The presence of astrocytic plaques showing an annular arrangement of tau-positive processes (**Figure 2B**), is required for the diagnosis. They represent tau deposition in distal astrocytic processes and are prominent in affected cerebral cortices and striatum. Tau-positive CBs are also widespread in cortices and white matter[18, 19, 45, 62]. Additional pathological features of argyrophilic grain disease or TDP-43 (TAR DNA-binding protein 43) immunoreactive lesions are found in excess of 40% of CBD cases[42, 72]**.**

Ultrastructurally the tau filaments extracted from CBD brains appear as 15nm wide straight filaments or ~20nm wide long periodicity ‘twisted ribbons’.

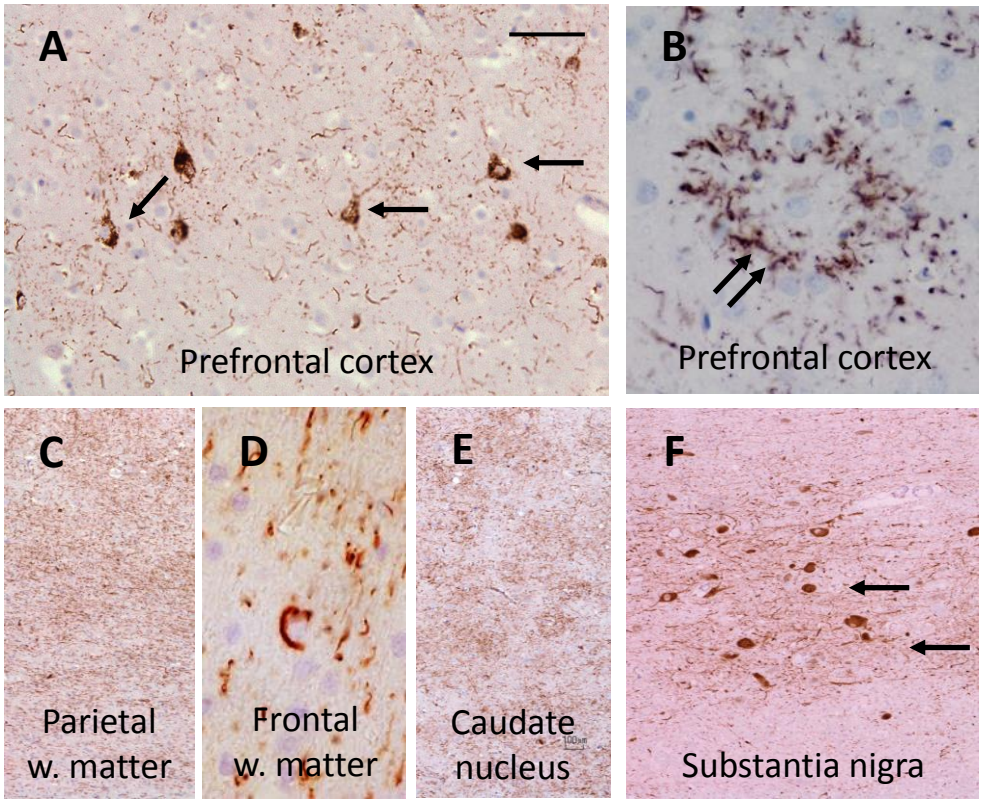

Figure 2 Tau immunohistochemistry in corticobasal degeneration. Arrows in **A** and **F** point to neurofibrillary tangles and pretangles. The presence of astrocytic plaques is required for the neuropathological diagnosis (**B**). There is abundant tau accumulation in axons and oligodendroglial processes in the subcortical white matter (**C**). Coiled bodies are also a feature (**D**). The striatum is one of the most severely affected structures showing abundant tau deposition (**E**). The neuronal loss in the substantia nigra is severe in advanced cases. In better preserved areas of the substantia nigra a significant proportion of the neurons possess tau inclusions (**F)**. Tau immunohistochemistry (AT8 antibody). Bar on A represents 90μm on **A**, **C, E** and **F**, 22,5μm on B and **D**. Parietal w. matter = Parietal white matter; Frontal w. matter

Clinical heterogeneity of CBD, described above, is well-recognised and attempts have been made to support this with neuropathological markers[43, 44, 47].

3.3 Genetics of progressive supranuclear palsy and corticobasal degeneration

A strong genetic link between the *MAPT* gene and development of PSP and CBD have been demonstrated[5, 33, 60]. Of the two common, tau haplotypes (H1 and H2), H1 is overrepresented in both PSP and CBD Caucasian patients[5]. Familial PSP and CBD have been described, but these should be considered cases of 'frontotemporal dementia and parkinsonism linked to chromosome 17' due to mutations in the *MAPT* gene (FTDP-17*MAPT*). Genome-wide association (GWA) studies in both diseases have demonstrated the same, previously unidentified genetic risk factors[34, 65].

3.4 Tau biochemistry in progressive supranuclear palsy and corticobasal degeneration

As in both PSP and CBD tau-positive neuronal and glial inclusions are the core neuropathological feature, they are classified as tauopathies. Tauopathy is an umbrella term used for a large group of diseases in which the presence of abundant tau inclusions is characteristic[23]. Due to alternative mRNA splicing of exons 2, 3 and 10 of the *MAPT* gene, located on 17q, there are six tau isoforms in the adult human brain. Based on the presence or absence of a fourth, 31 amino acid-long repeat region in the C-terminal, microtubule-binding domain of the tau protein encoded by exon 10, there are three tau isoforms with four repeat sequences (4R-tau) and three isoforms with three repeat sequences (3R-tau). Current classification of the tauopathies takes into consideration the tau isoform composition of the inclusions. In a large group of diseases with primarily neuronal tau-positive filamentous inclusions (NFTs), both 3R-tau and 4R-tau form the filaments (3R + 4R-tauopathies) while in others with inclusions in both neurons and glia, the filaments are predominantly composed of either 4R-tau (4R-tauopathies) or 3R-tau (3R-tauopathies) (**Figure 4**). In both PSP and CBD predominantly the 4R-tau isoforms form the filamentous inclusions with tau immunoblotting showing two strong bands at 68kDa and 64kDa (doublet pattern) with a third weak band at 72kDa[11, 68]. In contrast in 3R + 4R tauopathies such as Alzheimer's disease there is a triplet pattern with three strong bands are at 68kDa, 64kDa and 60kDa and an additional fourth, weak band at 72kDa (**Figures 3 and 4**). The presence of different C-terminal tau fragments differentiate PSP and CBD; a ~33kDA fragment has been

described in PSP and a 37kDA fragment in CBD[3]. The isoform composition of the tau inclusions also correlates with the ultrastructural features of the tau filaments (**Figure 4)**.

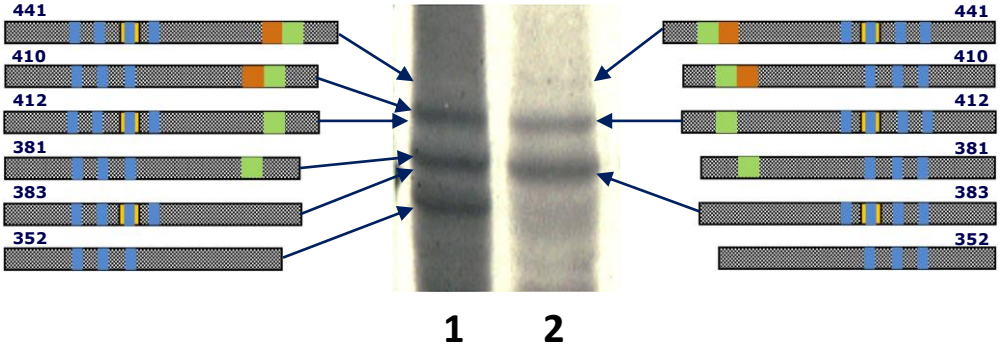

Figure 3 Illustration of tau western blot patterns in Alzheimer's disease (lane 1) and classical progressive supranuclear palsy (lane 2)

In Alzheimer's disease, there is a triplet electrophoretic migration pattern with three strong bands at 68kDa, 64kDa and 60kDa with a fourth weak band at 72kDa while in PSP a doublet pattern is characteristic (68kDa and 64kDa) with a third weak band at 72kDa. While all six tau isoforms contribute to Alzheimer's disease-tau, PSP-tau is mainly composed of 4R-tau (the fourth repeat region is represented by a blue square over yellow background.

Previously unpublished blots performed by Tamas Revesz

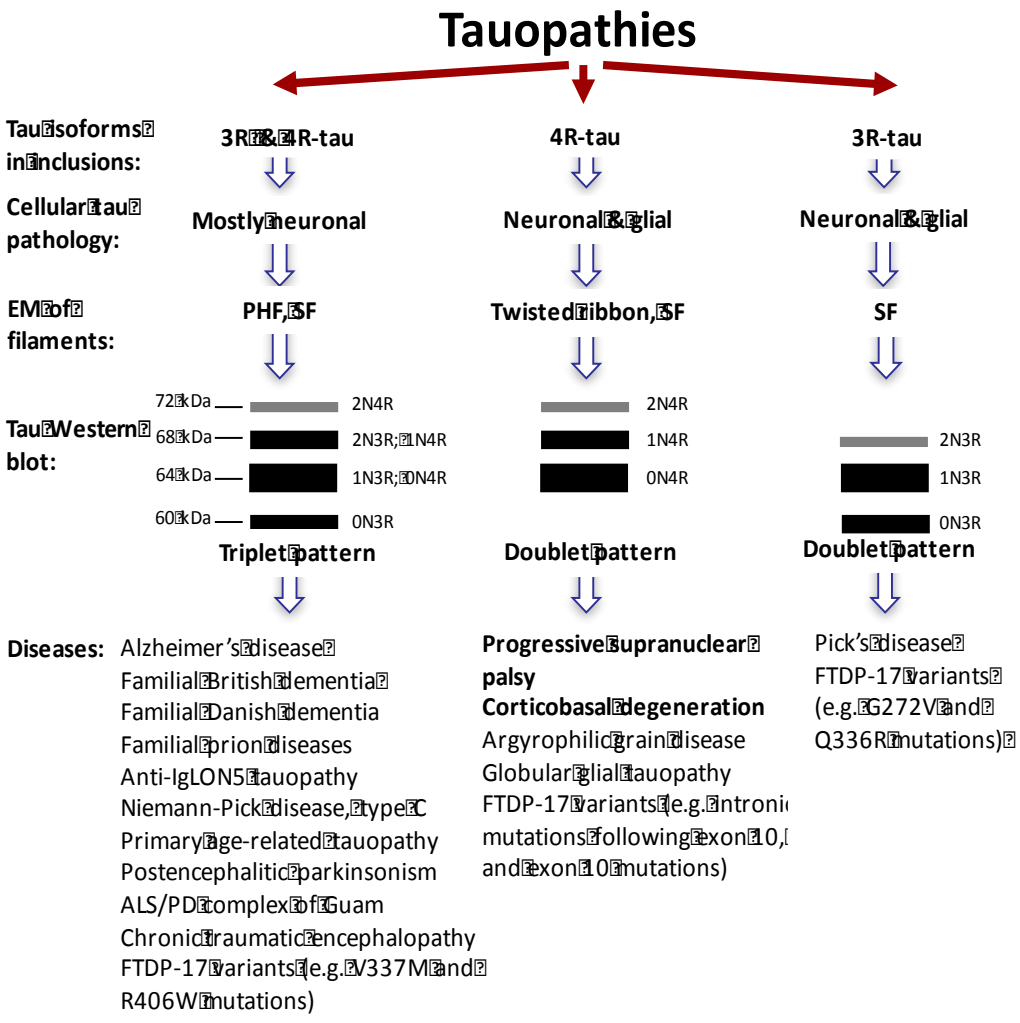

Figure 4 Examples of tauopathies

Tauopathies are classified on the basis of the tau isoform composition of the inclusions. The isoforms contributing to the bands seen on western blots are shown as N = N-terminal insert, 3R-tau = 3-repeat tau; 4R-tau = 4-repeat tau. ALS/PD complex = amyotrophic lateral sclerosis/parkinsonism complex; EM = electron microscopy; FTDP-17 = frontotemporal dementia and parkinsonism, linked to chromosome 17; PHF = paired helical filament, SF = straight filament

4. UNANSWERED QUESTIONS IN PSP AND CBD RESEARCH, RELEVANT FOR THE THESIS

In their seminal paper Steele, Richardson and Olszewski[70] predicted that atypical PSP, clinically deviating from the classical presentation, could emerge. Indeed, subsequently a few atypical cases with PSP post-mortem findings were documented in the literature and these included cases lacking SGP[16, 20] or presenting with severe dementia[7, 16], CBS[6, 15, 80] or pure akinesia[54].

Since its foundation in 1984, the QSBB has assembled a large cohort of PSP and CBD cases and by the mid-1990s cases with PSP pathology had been observed in which the clinical diagnosis of PSP was not suspected as the patients presented with an atypical syndrome, including a Parkinson's disease-like clinical phenotype[36] and many did not show signs of SGP[15]. In 1995 a systematic neuropathological study from the QSBB made an attempt to differentiate clinically atypical from typical PSP, but it failed to demonstrate distinct qualitative differences in the neuropathology between these two clinical groups of PSP[15].

Unlike in Alzheimer's disease, in which the patterns of progression of the neurofibrillary tangle pathology was successfully mapped over 25 years ago[9] and updated in 2006[8], the topographical spread of the tau pathology including the structures first affected by the disease process have remained enigmatic in both PSP and CBD.

5. AIMS OF THESIS

5.1 To show how our research group succeeded in differentiating clinically atypical PSP from typical PSP by using quantitative measures for mapping neuronal cell loss and tau pathology, by studying the biochemical profile of PSP-tau and employing genetic markers (**papers 1-4**).

5.2 To demonstrate how studying the neuropathology of 'incidental' (preclinical) cases could increase our understanding the patterns of progression of the tau pathology in CBD (**paper 5**).

6. INITIAL STUDIES AIMING TO DIFFERENTIATE TYPICAL AND ATYPICAL PSP (PAPERS 1 AND 2)

In view of the failure of the first comprehensive study from the QSBB to demonstrate neuropathological differences between clinically typical and atypical PSP in 1995[15] and my interest in PSP including understanding the neuropathological basis of SGP in this disease, I proposed a study of the nucleus raphe interpositus in typical PSP and atypical PSP defined by the absence of SGP (**paper 1**)[64].

Soon after the completion of the above study I established a successful collaboration with Professor Brian Anderton and his team at the Department of Neuroscience, Institute of Psychiatry, King's College, London with the aim of extending the neuropathological studies with biochemical analysis of PSP-tau in typical and atypical PSP cases. For this I learned the necessary techniques and carried out a significant part of the biochemical studies, published in **paper 2**[55] with help from Professor Brian Anderton and his team. For our second study we also determined the tau gene haplotypes in the same cohort of typical and atypical cases.

6.1 The nucleus raphe interpositus in typical and atypical PSP (paper 1)

Revesz et al.**:** The nucleus raphe interpositus in the Steele-Richardson-Olszewski syndrome (progressive supranuclear palsy). *Brain 119, 1137-1143, 1996*[64]

5.1.1 Background and research questions

Just a few years before this study was carried out, the importance of the omnipause neurons in the organisation of saccadic eye movements had emerged[12]. In order to learn the neuroanatomy of the omnipause neurons, in 1994 I spent a week in the laboratory of Professor Jean Buttner-Ennever at the University of Munich, who played a pivotal role in clarifying the neuroanatomy and normal function of the omnipause neurons located in the nucleus raphe interpositus[12, 31].

The premotor network responsible for saccadic eye movements are dependent on the normal function of two neuronal types located in the paramedian pontine reticular formation: a.) burst neurons, active before saccades and b.) pause neurons, active before and during saccades. Pause neurons include the omnipause neurons, located in the nucleus

raphe interpositus. They exert a tonic inhibition on the burst neurons, but pause before eye movements. The nucleus raphe interpositus with the omnipause neurons is located in the lower pons, just ventral to the medial longitudinal fascicle and dorsal to the gigantocellular nucleus. The rootlets of the of the 6th nerve represent the lateral border of the nucleus raphe interpositus (**Figure 5**). The cell soma of the glycinergic omnipause neurons is fusiform in shape and they possess well-developed horizontally oriented dendrites[12] (**Figure 6**).

In the study to be discussed here, we wished to investigate 1.) whether the nucleus raphe interpositus is affected in PSP and if yes 2.) whether such an involvement is different in atypical PSP without SGP compared with typical PSP.

6.1.2 Cases studied, methods used and the main findings of paper 1

We investigated 8 typical PSP cases with SGP, 5 atypical cases without SGP and 6 neurologically normal controls. The mean age at onset and mean disease duration are shown in **Table 1**. Patients without SGP were older than those with SGP (*P*=0.001, Student's *t* test), but there was no difference in the mean disease durations (*P*=0.662, Student's *t* test).

Table 1 Mean age at onset and disease duration in typical PSP with SGP and atypical PSP without SPG

Patient group	Mean age at onset years (SD, range)	Mean disease duration years (SD, range)
PSP with SGP	67.3 (5.4; 57-74)	5.9 (2.6; 2-10)
PSP without SGP	81.4 (5.8; 75-88)	6.6 (2.5; 4-9)
Normal controls	78.8 (5.3; 72-85)	N/A

N/A = Not applicable; PSP = progressive supranuclear palsy; SD = Standard deviation; SGP = supranuclear gaze palsy

In this study we determined the neuronal cell density (n/mm^3) of the omnipause neurons using an unbiased stereological method based on the principle of the optical disector[27]. NFT densities in the nucleus raphe interpositus were also determined. The extent of tissue shrinkage of the pontine tegmentum, containing the nucleus raphe interpositus, was estimated by calculating a ratio from measurements of the heights of the pontine tegmentum and base and these were used for calculating a 'correction factor' for each disease group.

Neuronal cell (ND/mm^3) and NFT densities (NFT/mm^2) are shown in **Table 2**. Neuronal cell density was significantly lower in typical PSP than in normal controls irrespective of whether tissue shrinkage was taken into consideration (*P*=0.001, Student's *t* test) or not (*P*=0.014, Student's *t* test). Having adjusted for tissue shrinkage, a difference in neuronal cell density was also apparent between the typical and atypical PSP cases (*P* = 0.016, Student's t test). Many of the omnipause neurons contained NFTs (**Figure 5**) and these were significantly more frequent in the typical PSP cases than in atypical PSP without SGP (P=0.011, Mann-Whitney U test). Furthermore, there was a moderate negative correlation between neuronal cell and NFT densities (Pearson's r =-0.5647; *P*=0.04).

Table 2 Densities of the omnipause neurons and neurofibrillary tangles

	Patient groups		
Data of morphometry	**PSP with SGP (typical PSP)**	**PSP without SGP (atypical PSP)**	**Normal control**
ND/mm^3 (SD, range)	1314.9 (492; 609.2-1998)	1627.4 (396.3; 1208.4-2248.4)	1888 (211; 1488-2102)
ND/mm^3, ADJ (SD, range)	**946.8** (354.3; 438.6-1438.6)	**1594.8** (388.4; 1184.2-2203.4)	**1888** (211; 1488-2102)
NFT/mm^2 (SD, range)	**13.5** (8.3; 1.8-29.2)	**2.5** (1.9; 0-5.2),	**0**

ADJ = adjusted; ND = neuronal cell density; NFT = neurofibrillary tangle; SD = standard deviation; PSP = progressive supranuclear palsy; SGP = supranuclear gaze palsy

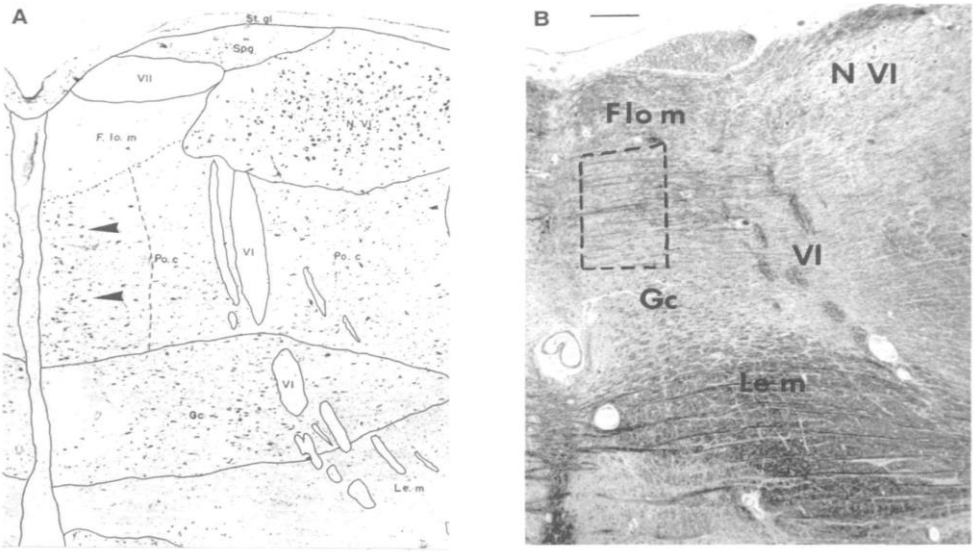

Figure 5 The nucleus raphe interpositus containing the omnipause neurons is located in the paramedian tegmentum of the caudal pons

F lo m = medial longitudinal fascicle, N VI = 6th nerve nucleus, Gc = gigantocellular nucleus, Le m = medial lemnisc, VI = rootlets of the 6th nerve.

A: Olszewski and Baxter: Cytoarchitecture of the Human Brain Stem, 2nd ed. Karger, Basel, 1982. **B**: Luxol fast blue cresyl violet.

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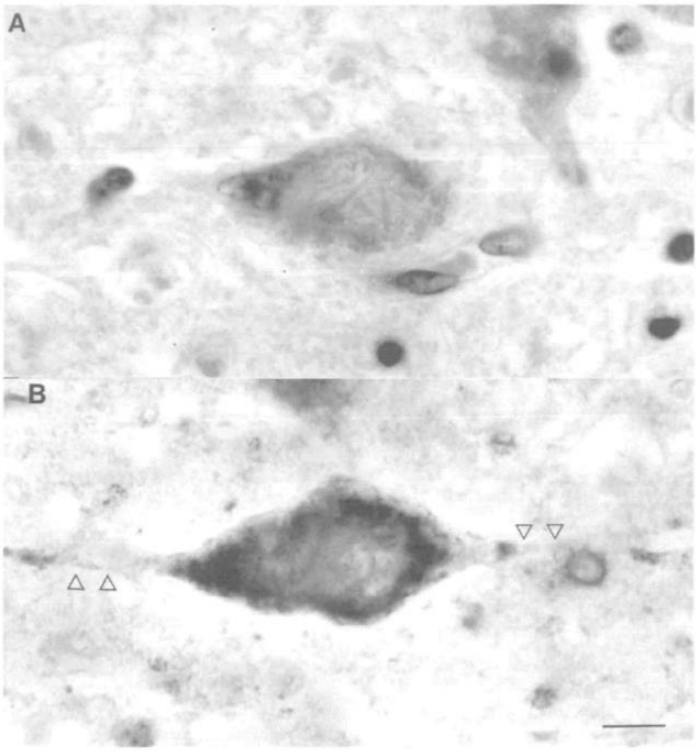

Figure 6 Omnipause neurons in the nucleus raphe interpositus
The omnipause neurons are fusiform in shape and have horizontally oriented processes (open arrowheads). The two neurons shown contain neurofibrillary tangles. **A**: haematoxylin and eosin preparation, **B**: tau immunohistochemistry. Bar on **B** represents 5μm

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6.2 Biochemical and genetic heterogeneity in typical and atypical PSP variants (paper 2)

Morris et al.: Pathological, clinical and genetic heterogeneity in progressive supranuclear palsy**.** Brain 125:969-975, 2002[55]

6.2.1 Background to research and questions arising

In our study on the nucleus raphe interpositus (**paper 1)**[64] we could differentiate typical PSP from a PSP variant by morphometry employing stereological principles. In the second study, we investigated the hypothesis that the western blot profile of the PSP-tau and the distribution of the H1 and H2 tau (*MAPT*) gene haplotypes are different in typical PSP and the atypical PSP variants.

6.2.2 Cases studied, methods and main findings of paper 2

We investigated a cohort of 15 atypical and 11 typical PSP cases, which were available in the archives of the QSBB. Clinically typical cases met established diagnostic criteria including the presence of SGP[73]. The atypical group was heterogeneous; some cases presented with a Parkinson's disease-type syndrome and in a minority of the atypical cases SGP was absent. There was no difference in the mean age of onset (P=0.9, Student's t test) and mean age of death between the typical and atypical groups (P=0.09 Student's t test) (**Table 3**). All cases were confirmed to have PSP neuropathological changes with additional Alzheimer's disease-type pathology in 19 of the 26 cases.

For tau biochemistry insoluble tau was extracted from the frozen half of PSP brains using a previously described method[28]. For genetic analysis DNA was extracted from frozen brain tissue samples and the tau haplotype analysis was carried out by using standard methods[5].

In both the typical and atypical cases the insoluble tau-enriched protein fraction was probed with SDS-PAGE (sodium dodecyl sulphate–polyacrylamide) gel electrophoresis and two anti-tau antibodies, the phosphorylation independent TP70, recognising C-terminal amino acids 428-441[10] and the phosphorylation dependent PHF-1 antibody detecting pSer396/404 epitopes[26].

The majority of the clinically typical cases (73%) showed the characteristic PSP-doublet electrophoretic migration pattern with two strong bands at 68kDa and 64kDa and a third

Table 3 Comparison between clinically typical and atypical PSP

PSP	n	Mean age at onset (years)	Mean age at death (years)	H1/H1 haplotype (%)	Doublet band (%)
Atypical	11	66.3	76.3	73.3	33.3
Typical	15	66	71.4	100	72.7

PSP = progressive supranuclear palsy

weak band at 72kDa[21] (**Figure 7**, lanes 2,6-8) as compared with only one third (33%) of the atypical cases showing this pattern. The atypical PSP-tau western blot patterns included a triplet pattern with three strong bands at 68, 64kDa and 60kDa with a fourth weak band at 72kDa, as seen in Alzheimer's disease (**Figure 7**, lane 3), and a pattern consisting of 6 to 8 protein bands (**Figure 7**, lane 4), which migrated similarly to the 6 recombinant isoforms (**Figure 7**, lane 5).

All cases, clinically classified as typical, were homozygous for the H1/H1 PSP susceptibility *MAPT* genotype compared with 73% of the atypical cases.

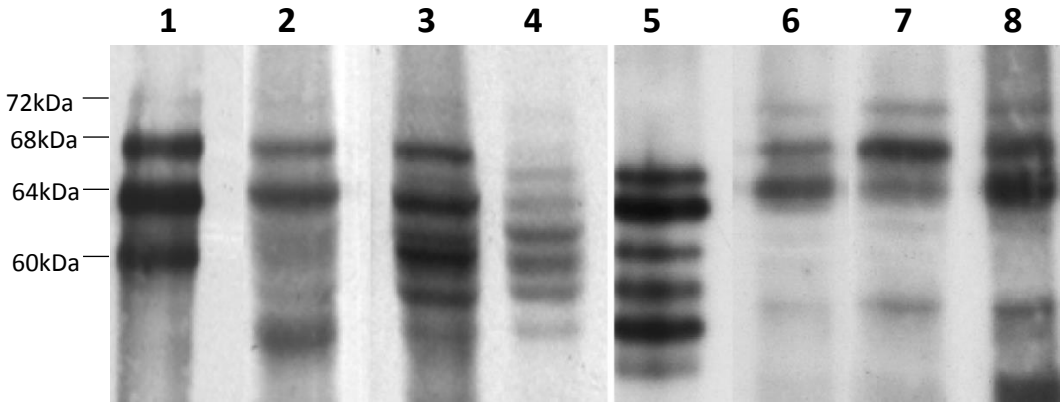

Figure 7 Western blots in typical and atypical progressive supranuclear palsy
Electrophoretic migration patterns of insoluble tau probed with the TP70 (lanes 1-5) and PHF-1 (lanes 6-8) antibodies. Lane 1: PHF-tau triplet pattern in Alzheimer's disease, lane 5: six recombinant tau isoforms. Typical PSP-tau doublet migration pattern: lanes 2, 6-8; PHF-tau-like pattern lane 3; atypical pattern with 6 to 9 bands: lane 4.

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6.3 Conclusions of papers 1 and 2

Using stereological tools for morphometry in our study reported in **paper 1**[64]**,** we demonstrated for the first time that the omnipause neurons located in the nucleus raphe interpositus are severely affected by the PSP disease process. We demonstrated an ~50% loss of these neurons in typical PSP compared with normal controls and ~30% greater loss compared with the atypical cases. Furthermore, the observation of a greater NFT load in the typical cases was also consistent with the notion that the nucleus raphe interpositus is differentially affected in typical and atypical PSP with the atypical group being defined by the absence of SGP.

In **paper 2**[55] we investigated whether typical and atypical PSP have different tau biochemical profiles and/or show differences in the distribution of the H1 and H2 *MAPT* gene haplotypes. We found that while the PSP genetic susceptibility tau gene haplotype and the characteristic, previously described PSP-tau doublet electrophoretic migration pattern were strongly associated with typical PSP, the atypical cases less frequently possessed *MAPT* H1/H1 genotype and often showed an atypical western blot pattern, which is consistent with an increased contribution of 3R-tau isoforms to PSP-tau in the atypical cases.

7. COMPARISON OF CLINICALLY WELL-CHARACTERISED ATYPICAL PSP WITH TYPICAL PSP (PAPERS 3 AND 4)

7.1 The concept of Richardson's syndrome (PSP-RS), PSP with parkinsonism (PSP-P) and PSP with pure akinesia with gait freezing (PSP-PAGF)

Although early systematic investigations by our research group[55, 64] and also by others[52] raised the profile of atypical PSP, these studies, due to lack of satisfactory clinical definition of the atypical PSP variants, could not provide reliable neuropathological criteria of atypical PSP[17]. This situation fundamentally changed when, using hierarchical cluster analysis, our research group could separate reliably three clinical phenotypes of PSP in a large cohort of neuropathologically confirmed and clinically well-documented cases (n=103)[77]. The largest group was represented by cases with classical clinical presentation, for which the term Richardson's syndrome (PSP-RS) was introduced after J. Clifford Richardson, who first described the clinical signs and symptoms of PSP. The second group was represented by cases with a Parkinson's disease-like presentation with asymmetry at disease onset, tremor and slight initial response to levodopa (PSP-parkinsonism or PSP-P). A third small group designated as PSP-pure akinesia with gait freezing (PSP-PAGF) was also identified[79]. PSP-RS patients were younger at both disease onset and death, had shorter disease duration than patients with PSP-P. PSP-RS patients were more frequently males than females while the sex distribution was even in PSP-P. Similar to our previous study described in **paper 2**, biochemical investigation of PSP-tau showed a greater contribution by 3R-tau to the insoluble fraction of the tau protein in PSP-P than in PSP-RS while the effect of the H1/H1 PSP susceptibility genotype was greater in PSP-RS than in PSP-P[77].

7.2 Differences in tau-burden and its neuroanatomical distribution differentiate PSP-RS and PSP-P (paper 3)

Williams et al.: Pathological tau burden and distribution distinguishes progressive supranuclear palsy-parkinsonism from Richardson's syndrome. Brain 130:1566-1576, 2007[78]

7.2.1 Background and research questions

Although following the above described seminal study by Williams et al. 77] from the QSBB, the existence of PSP-P, and subsequently PSP-PAGF as clinical variants were independently

confirmed by other research groups[2, 17, 38, 58], the question remained unanswered whether the biological differences that may define these variants (i.e. a greater contribution by 3R-tau to PSP-tau in PSP-P, the greater effect of the H1/H1 PSP susceptibility genotype in PSP-RS and other unknown factors), also manifest in differences in the severity and/or the anatomical distribution of the tau pathology. Our study, published in **paper 3**[78] wished to answer these questions.

7.2.2 Cases studied, methods and main findings of paper 3

Forty two pathologically proven cases (26 males, 16 females) (22 PSP-RS, 14 PSP-P, 6 PSP-PAGF) were selected from the original cohort that was used for identifying the three clinical variants (see above)[77]. There was no difference in the prevalence of secondary pathologies (amyloid-β (Aβ) plaques, cerebral amyloid angiopathy (CAA), argyrophilic grains, Lewy body pathology and cerebrovascular disease) between the groups.

We used the AT8 anti-tau antibody, recognising pSer202/Thre205, for tau immunohistochemistry and an unbiased morphometric method for the quantitation of the different tau-positive lesions. Accordingly, the severity of NFT, Th, tufted astrocyte and CB pathologies was determined in 17 brain regions which are known to be affected by the PSP disease process. The continuous data were converted into a 5-tiered grading system (grades 0 to 4) in each region; tau-positive Ths and CBs were pulled into a single measure (CB+Th).

The 'total tau-load' (Σ of grades for all lesion types in all regions) was higher in PSP-RS than in PSP-P and PSP-PAGF (*P*=0.002) (**Figure 8**). The mean 'regional tau-load' determined in 17 brain regions was, in general, also higher in PSP-RS than PSP-P or PSP-PAGF.

As part of this project a simplified approach for the assessment of the tau pathology was also developed. It was found that when measures of CB+Th pathologies alone (omitting measures of NFTs and tufted astrocytes from the analysis) were graded semiquantitatively as 0 – 4 in three anatomical areas (substantia nigra, caudate and cerebellar dentate nucleus), these simplified measures could efficiently capture the 'total tau-load', which had

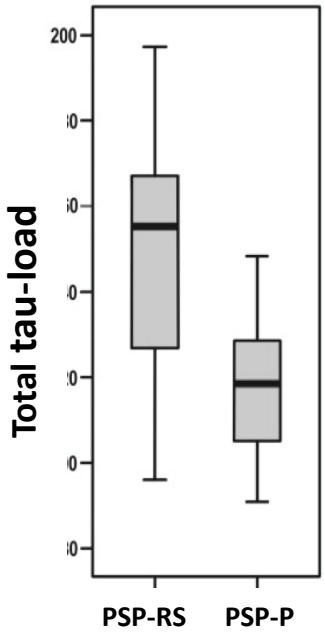

Figure 8 'Total tau-load' in typical (PSP-RS) and atypical progressive supranuclear palsy (PSP—P)

The 'total tau-load' (Σ of grades for all lesion types in all areas) in PSP-RS is significantly greater than that in PSP-P, median (Mann-Whitney U test, *P*=0.002) and interquartile ranges (the small PSP-PAGF group was excluded from the analysis). PSP = progressive supranuclear palsy; PSP-RS = PSP-Richardson's syndrome; PSP-P = PSP-parkinsonism.

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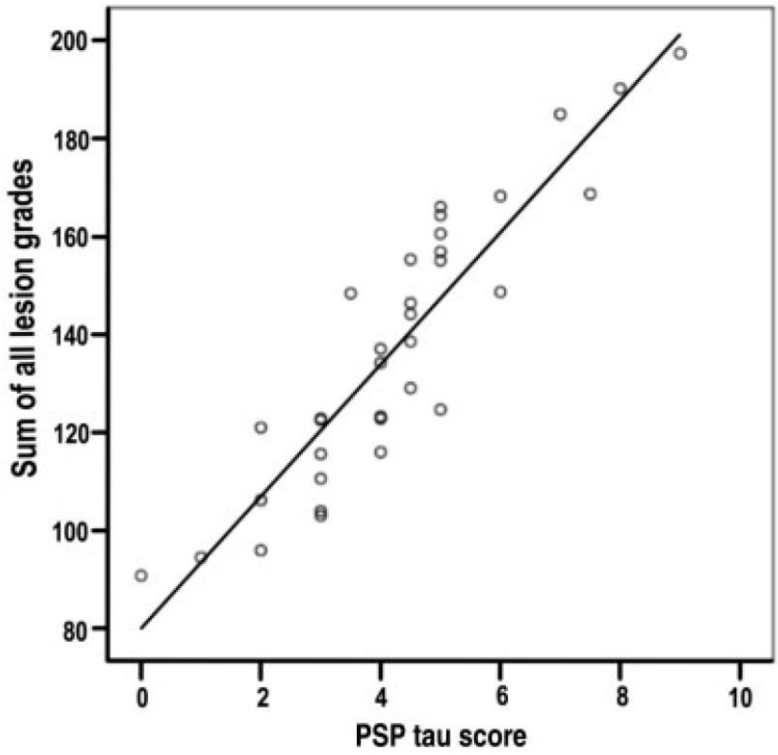

Figure 9 Correlation between PSP-tau scores and sum of all lesion grades (Spearman's rho 0.93, *P* < 0.001)

PSP = progressive supranuclear palsy

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been determined by time-consuming assessment of the tau pathology in 17 brain regions. This simple semiquantitative measure was designated as 'PSP-tau score', which ranged between 0 to 12 (grades 0-4 in each of the 3 regions investigated) for each case. The usefulness of the 'PSP-tau scores' is shown by the excellent correlation between the 'PSP-tau scores' and the previously determined 'total tau load' (Σ of grades for all lesion types in all 17 brain regions) (**Figure 9**).

Using the 'PSP-tau scores' as surrogate markers of the severity of the cerebral tau-burden important and novel clinicopathological correlations were established; no PSP-P cases showed a 'PSP-tau score' higher than 5 (median 3) while the median for the PSP-RS group was 5, and that, similar to the 'total tau-score', the 'PSP-tau scores' could convincingly establish a difference in the tau-load between the PSP-RS and the PSP-P groups (Mann-Whitney U test, $P<0.001$) (**Figure 10**, for comparison also see **Figure 8**).

Cases were also grouped using the 'PSP-tau scores' (PSP-tau score = 0-1; PSP-tau score = 2-3; PSP-tau score = 4-5; PSP-tau score 6-7; PSP-tau score >7). Furthermore, median values of CB+Th determined previously by morphometry, for each of the 17 regions were calculated for each 'PSP-tau score' category (the relationship between the 'PSP-tau scores' and CB+Th median values is illustrated in **Figure 11**). This approach revealed that with the increase of the 'PSP-tau scores' there is a gradual topographical expansion and an increase in the severity of the tau pathology (**Figure 11**). In brief, the tau pathology initially is predominantly restricted to pallido-luyso-nigral structures with mild involvement of the premotor cortex with lower brainstem structures showing no or mild tau pathology (**Figure 11B**). In cases with higher PSP-tau scores the tau pathology gradually extends into the striatum, pontine nuclei, cerebellum (**Figure 11C**) and other cortical regions (**Figure 11D**). In cases with 'PSP-tau scores' 5-6 and >7, severe involvement of the subthalamic nucleus, substantia nigra, internal globus pallidus, neocortical areas, pontine nuclei and cerebellar structures is characteristic and an increase in the severity of the cortical tau pathology is recognised.

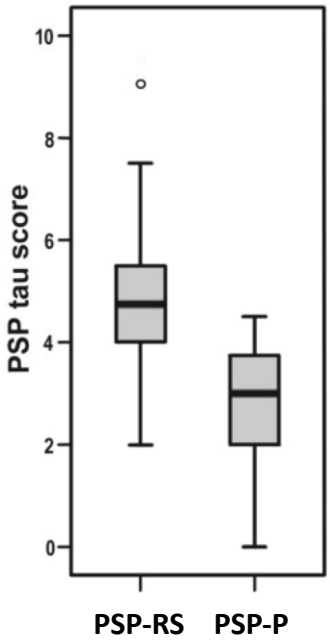

Figure 10 Using PSP-tau scores confirms a greater tau-load in PSP-RS than in PSP-P

PSP-tau scores according to clinical group, median (Mann-Whitney U test, $P < 0.001$), and interquartile ranges. PSP = progressive supranuclear palsy; PSP-RS = PSP-Richardson's syndrome; PSP-P = PSP-parkinsonism.

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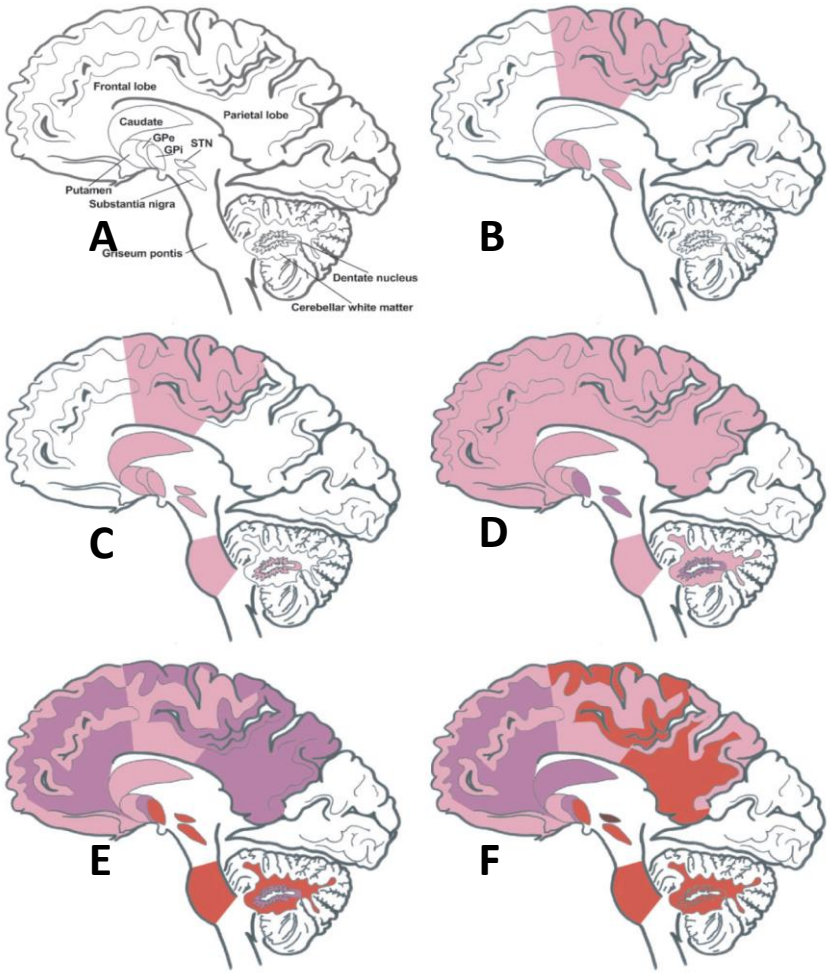

Figure 11 Neuroanatomical distribution of the CB+Th (coiled body + thread) pathology (colour/grade) according to the PSP-tau scores

Severity of CB+Th pathology: pink = grade 1; purple = grade 2; red = grade 3; brown = grade 4. **A** = anatomical structures/control; **B** = PSP-tau score 0-1; **C** = PSP-tau scores 2-3; **D** = PSP-tau score = 4-5; **E** = PSP-tau score 6-7; **F** = PSP-tau score >7. No PSP-P case had a PSP-tau score higher than 5 illustrated by **D**. CB + Th pathology = coiled body + neuropil thread pathology; PSP = progressive supranuclear palsy.

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7.3 PSP cases clinically presenting with corticobasal syndrome (PSP-CBS) is defined by a characteristic distribution of the tau pathology (paper 4)

Ling et al.: Characteristics of progressive supranuclear palsy presenting with corticobasal syndrome: a cortical variant. Neuropathol Appl Neurobiol 40:149-163, 2014[46]

7.3.1 Background and research questions

The term CBS is used to describe a characteristic set of clinical signs including apraxia in one hand, alien limb phenomenon, cortical sensory loss, dystonia and levodopa-unresponsive rigidity. Although initially it was described as the clinical manifestation of CBD, subsequently several other diseases, including PSP[6, 15, 50, 55, 74, 80] have been documented as its cause.

PSP-CBS, as a rare clinical PSP variant, emerged in the early 1990s[6, 15, 80]. Two previous studies investigated systematically 3 and 5 cases of this variant[6, 74] and concluded that PSP-CBS is associated with increased cortical tau pathology. However, neither of these two studies provided an insight into the overall distribution of the tau pathology in PSP-CBS, which we wished to address in the study, published in **paper 4**[46].

7.3.2 Cases studied, methods and main findings of paper 4

227 neuropathologically confirmed PSP cases were archived in the QSBB between 1988 and 2010, of which 9 had the clinical diagnosis of CBS/CBD (3.9% of all PSP cases). An additional case was obtained for our investigations through collaboration from the University of Nottingham, making our study the largest neuropathological investigation of this variant to date. 10 PSP-RS cases, matched for disease duration and age at death were used as controls. There was no difference in the mean age of onset, mean age at death and mean disease duration between the two groups (**Table 4)**.

Using tau immunohistochemistry with the AT8 antibody, the tau pathology (NFTs, PreTs, tufted astrocytes, Th, CBs) was quantitated in 15 brain regions. In each area, images were captured from 10 random microscopic fields, which were analysed by an image analysis software (Image-Pro; Media Cybernetics, Inc., Roper Industries, Rockville, USA). The

stereological tool, 'areal fraction' was used as a measure of tau-burden in each anatomical region. From the measurements in all 15 cerebral areas, the 'total tau-load' was calculated for each case; the tau-load in all the cerebral cortical areas provided the 'cortical tau-load' and in the basal ganglia the 'basal ganglia tau-load'. After initial data analysis, NFTs, PreTs, tufted astrocytes, CBs were also quantitated in three anatomical regions (posterior frontal cortex, anterior frontal cortex and caudate nucleus), which were the regions that had been found to show robust differences in the tau-load between PSP-RS and PSP-CBS.

Table 4 Demographic features of PSP-CBS and PSP-RS cases

	PSP-CBS	**PSP-RS**	***P* values (Student's *t* test)**
Mean age at disease onset	65.9 $\pm$ 8.0	65.9 $\pm$ 7.9	0.98
Mean age at death	73.4 $\pm$ 6.4	74.4 $\pm$ 6.5	0.72
Mean disease duration	7.5 $\pm$ 2.7	8.6 $\pm$ 4.4	0.51

Using a four-tiered grading system neuronal cell loss was determined semiquantitatively in the substantia nigra and subthalamic nucleus.

PSP-tau in PSP-CBS and in PSP-RS was studied by western blotting and *MAPT* gene H1 and H2 haplotypes were also determined.

Pathological review confirmed that both the PSP-RS and PSP-CBD groups met established neuropathological diagnostic criteria of PSP[19, 30, 62]. There was no difference in neuronal cell loss in the subthalamic nucleus between the two groups (χ^2 test, $P > 0.05$), but in the substantia nigra the neuronal cell loss was more severe in the PSP-RS group than in the PSP-CBS cases (χ^2 test, $P = 0.018$).

Analysis of the median 'regional' tau-load showed that in the prefrontal (Mann-Whitney U test, P=0.003) and posterior frontal cortices (Mann-Whitney U test, P<0.001) and parietal subcortical white matter (Mann-Whitney U test, P=0.001) it was greater in PSP-CBS than in PSP-RS, while the median 'regional' tau-load in the caudate (Mann-Whitney U test, P<0.001)

and the subthalamic nucleus (Mann-Whitney U test, *P*<0.001) was greater in PSP-RS than in PSP- CBS (**Figure 12**). It is of note that, although the 'total tau-load' (Σ of 'regional tau-load' in all 15 structures studied) was not different in the two disease groups (Mann-Whitney U test, *P*=0.176) (**Figure 13A**), the 'cortical tau-load' (Σ of 'regional tau-load' in all 7 cortical regions, studied) was significantly greater in PSP-CBS than in PSP-RS (Mann-Whitney U test, *P*<0.001) (**Figure 13B**). In contrast, the 'basal ganglia tau-load' (Σ of 'regional tau-load' in the caudate nucleus, putamen, globus pallidus and subthalamus) was significantly greater in PSP-RS than in PSP-CBS (Mann-Whitney U test, *P*=0.003) (**Figure 13C**). Quantitation of the different tau lesion types confirmed that all lesion types were more frequent in the posterior frontal cortex in PSP-CBS than in PSP-RS (Mann-Whitney U test, *P*<0.001) while all tau-positive lesion types were more frequent in the caudate nucleus in PSP-RS than in PSP-CBS (Mann-Whitney U test, $P < 0.001$).

Protein analysis of PSP-tau by western blotting demonstrated the characteristic doublet pattern with two strong bands at 68kDa and 64kDa and a faster migrating band at ~33kDa as previously described[3, 75]. There was no difference in the distribution of the H1/H1 and H1/H2 genotype in the two PSP variants, either (χ^2 test, *P*=0.21).

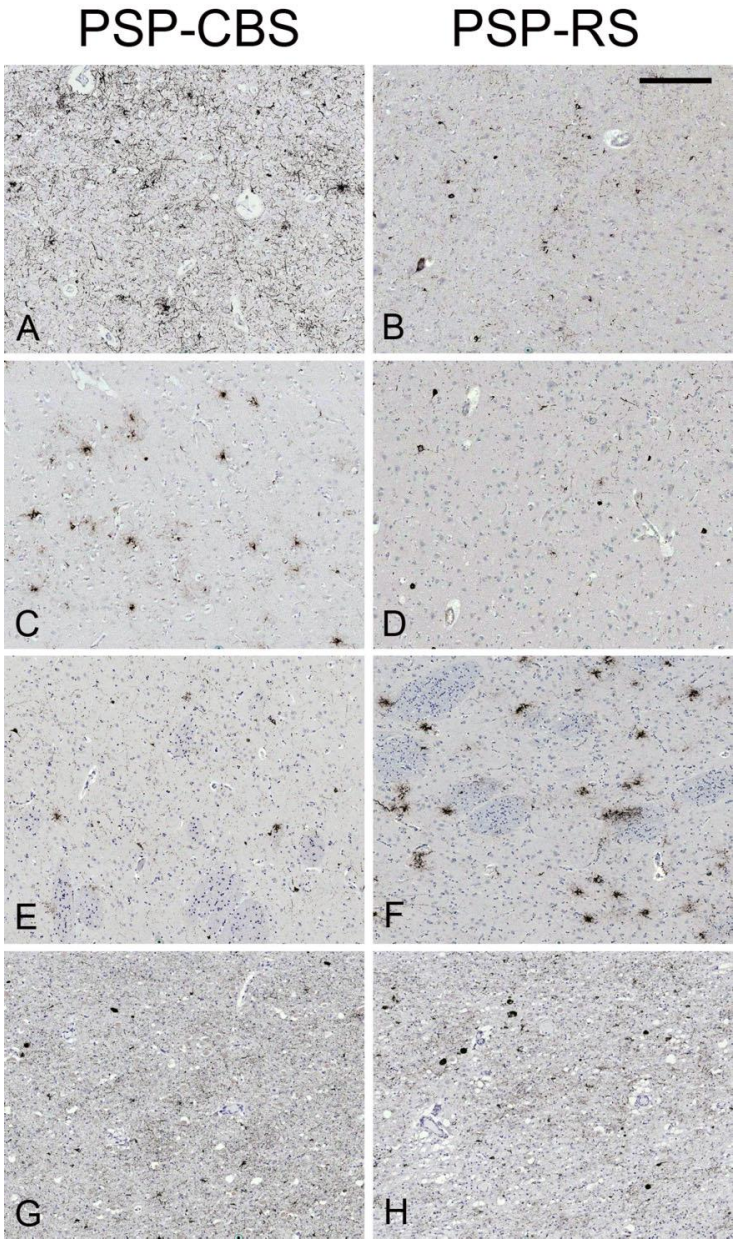

Figure 12 The tau-load is greater in the posterior frontal (**A**) and prefrontal cortex (**C**) in PSP-CBS than in the corresponding areas in PSP-RS (**B** and **D**). In contrast, the tau-load is greater in the caudate (**F**) and subthalamic nucleus (**H**) in PSP-RS than in PSP-CBS (**E** and **G**).

Tau immunohistochemistry (AT8 antibody). Bar on **B** represents 225 microns in all panels. PSP = progressive supranuclear palsy; PSP-CBS = PSP-corticobasal syndrome; PSP-RS = PSP-Richardson's syndrome.

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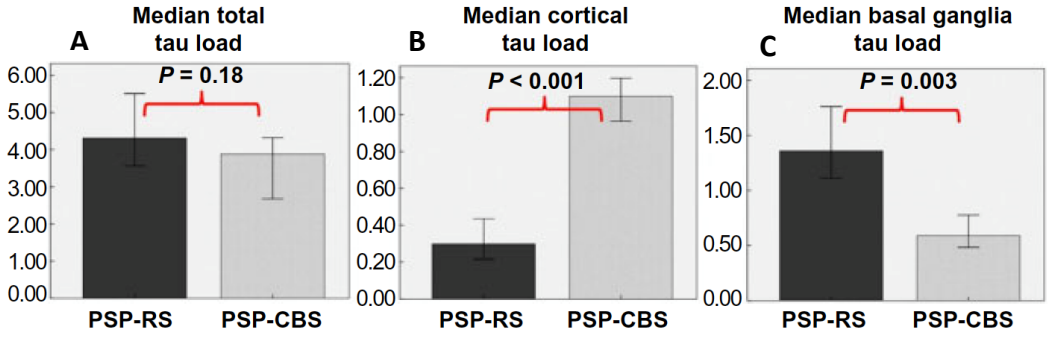

Figure 13 'Total', 'cortical' and 'basal ganglia tau-load' in PSP-CBS and PSP-RS
The median 'total tau-load' is similar in PSP-CBS and PSP-RS (**A**). While the median 'cortical tau-load' is significantly greater in PSP-CBS than in PSP-RS (**B**), the median 'basal ganglia tau-load' is significantly higher in PSP-RS than in PSP-CBS (**C**) (Mann-Whitney *U*-test). This indicates a shift of the tau-burden from the basal ganglia to the cerebral cortices in PSP-CBS. PSP = progressive supranuclear palsy; PSP-CBS = PSP-corticobasal syndrome; PSP-RS = PSP-Richardson's syndrome.

7.4 Conclusions of papers 3 and 4

In the studies described in **papers 3** and **4** we had the opportunity to investigate clinically well-characterised PSP variants, PSP-P, PSP-PAGF and PSP-CBS in comparison with PSP-RS. We showed that in each of the PSP variants the severity and distribution of the tau pathology deviates from those that can be found in the classical form of the disease (PSP-RS). In **paper 3**[78]**,** in which we determined the cerebral tau-load by analysing the tau pathology in 17 brain regions in 42 cases, we could demonstrate that the tau-burden in the PSP-P and PSP-PAGF variants is less severe and more restricted in its anatomical distribution than in PSP-RS. These findings have been validated by subsequent studies carried out by other research groups[17, 19, 38].

The main finding of our **paper 4**[46] is that, although the total tau-burden is similar in PSP-RS and PSP-CBS, there is a significant difference in the cortical and basal ganglia tau-load between these two PSP variants. This finding indicates a shift in the tau-burden from the basal ganglia to the cerebral cortex in PSP-CBS, which could be responsible for the clinical signs indicative of cortical dysfunction in this variant[6, 74]. Biochemical and genetic investigations of PSP-CBS, carried out in comparison with PSP-RS, demonstrated no difference in the PSP-tau electrophoretic migration pattern or the distribution of the H1 and H2 haplotypes between the PSP-CBS variant and the classical/typical form of the disease.

8. PATTERNS OF DISEASE PROGRESSION IN CBD: LESSONS LEARNED FROM THE STUDY OF 'INCIDENTAL' (PRECLINICAL) CASES (paper 5)

Ling et al.: Astrogliopathy predominates the earliest stage of corticobasal degeneration pathology. Brain 139:3237-3252,2016[47]

8.1.1 Background and research questions

Previous studies have provided information about the neuropathological progression of tauopathies, including Alzheimer's disease[8], Pick's disease[37] and argyrophilic grain disease[66]. Such investigations identified, at least in Alzheimer's disease and argyrophilic grain disease the anatomical areas that are first affected by the disease process. Furthermore, these studies also demonstrated that the specific tau pathologies, which characterise these conditions, affect with time, more and more cerebral areas and that progression stereotypically takes place in distinct hierarchical stages. However, patterns of progression of and, in particular, the structures first affected by tau pathology in PSP or CBD are not clearly understood and these questions in relation to CBD are addressed in **paper 5**[47].

8.1.2 Cases studied, methods and main findings

With the help of a grant I received from the Karin & Sten Mortstedt CBD Solutions AB, Stockholm, Sweden and through collaboration with several UK, European and US brain banks, my research group had the opportunity to collect over 120 neuropathologically proven CBD cases, which is one of the largest case series which has been studied to date. This has allowed us to initiate a large-scale, still on-going study, which aims to decipher the enigma of disease progression, patterns of spread of the tau pathology and the neurobiological basis of disease variants in CBD. As part of this project, we investigated 4 'incidental', clinically well-documented (preclinical) CBD cases, which provided data relevant for understanding where the disease process may start, and how the tau pathology may progress in the earliest disease stages. Three of the 4 cases have been reported in **paper 5**[47]. In this study, we also included 6 end-stage cases (3 CBD-CBS and 3 CBD-RS) as controls (for demographic data, clinical diagnosis see **Table 5**).

'Tau-load' was determined in 20 brain regions whose involvement is characteristic during the CBD disease process. For this, the slides immunostained with the AT8 antibody, were digitised and 'areal fractions' (area occupied by tau-positive structures/total tissue area) were computed with the help of an image analysis software (Definiens Developer 2.3). In addition to determining the 'regional tau-load' in the 20 brain regions, the 'total tau-load' (Σ of all tau-positive lesions in all regions), the 'cortical tau-load' (Σ of all tau-positive lesions in all cortical regions) and the 'basal ganglia tau-load' (Σ of all tau-positive lesions in the caudate nucleus, putamen, globus pallidus, subthalamus) were also calculated. Furthermore, the severity of the tau-positive cellular pathology, including that of NFT, PreT, astrocytic plaque

Table 5 Demographic data of preclinical and end-stage CBD cases

Case No.	Age at death (years)	Disease duration (years)	Clinical diagnosis
Case 1 (preclinical)	63	N/A	Tourette's syndrome
Case 2 (preclinical)	89	N/A	US Ageing Project
Case 3 (preclinical)	76	N/A	Kidney transplant
Case 4 (CBD-CBS)	78	8	CBS
Case 5 (CBD-CBS)	72	5	CBS
Case 6 (CBD-CBS)	73	7	CBS
Case 7 (CBD-RS)	68	5	RS
Case 8 (CBD-RS)	64	4	RS
Case 9 (CBD-RS)	66	4	RS

CBS = corticobasal syndrome; N/A = not applicable; RS = Richardson's syndrome

and CB pathologies, was determined in the anterior/prefrontal and posterior frontal, parietal and temporal cortices and white matter, medial temporal lobe structures, basal ganglia, brainstem and cerebellar structures. The severity of the tau-positive thread pathology was determined semiquantitatively using a four-tiered grading system.

Using a four-tiered grading system cell loss was also quantitated in the substantia nigra. The presence or absence of argyrophilic grains, Aß-positive plaque and Lewy body pathologies, CAA and cerebrovascular disease was documented.

The *MAPT* gene (exons 10-13) was screened for mutations; the *MAPT* haplotypes were determined by H1/H2-tagging SNP rs1052553.

Our data showed that the mean 'regional tau-load' in the group with 'incidental' CBD was less than that in the control end-stage cases with statistically significant difference identified in 16 selected regions (P<0.05). Both the 'total tau-load' (*P*=0.04) and the 'basal ganglia tau-load' (*P*=0.001) was significantly less in the 'incidental' than in the control group with end-stage CBD cases. A remarkable difference in the distribution of the tau pathology was noted in the frontal lobe; the tau deposition was characteristically severe in the posterior frontal region in the end-stage cases while this was far more prominent in the prefrontal cortical region than in the posterior frontal area in the 'incidental' cases (**Figure 14**). This was underlined by quantitative data, the mean anterior-to-posterior frontal tau-load ratio was 16.04 in the 'incidental' CBD group and 1.36 in the end-stage CBD group.

Quantitative analysis of cellular lesion types revealed important differences in that neuronal lesions were about four times more numerous than astrocytic plaques in the cortical regions (prefrontal, posterior frontal, parietal and temporal cortices) in the end-stage cases while the frequency of astrocytic plaques and neuronal lesions was similar in 'incidental' CBD (**Figure 15**) (mean neuronal to astrocytic plaque ratio was 4.20 in the end-stage and 0.91 in the preclinical cases ($P < 0.001$, χ^2 test)).

The neuronal cell loss in the substantia nigra was graded as absent or mild in the 'incidental' CBD cases while this ranged from moderate to severe in the end-stage cases (P=0.03, χ^2 test).

Tau-positive grains and mild cortical Aβ pathology were observed in one 'incidental' case each.

Analysis of exons 10-13 of the *MAPT* genes revealed no mutation; two cases had the H1/H1 *MAPT* haplotype (Cases 1 and 3) while the third case (Case 2) had H1/H2 haplotype.

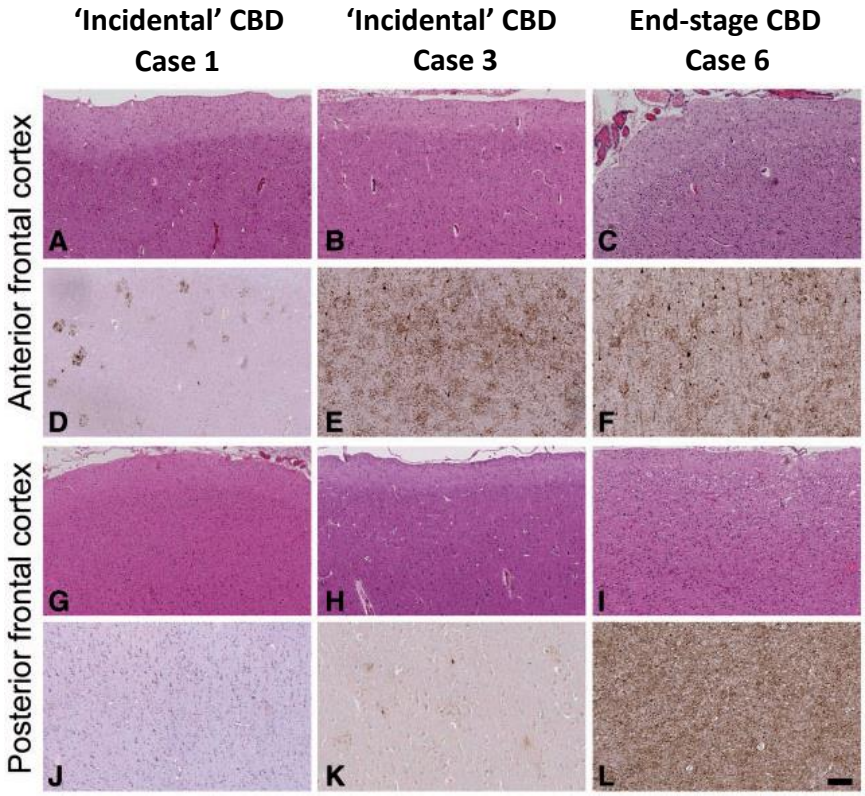

Figure 14 Tau pathology in the anterior and posterior frontal cortex in 'incidental' and end-stage CBD

In the 'incidental' cases the tau pathology is more severe in the anterior than in the posterior frontal cortex while in end-stage CBD both the anterior frontal and posterior frontal regions show severe tau deposition. The astrocytic plaques are a predominant lesion type in 'incidental' CBD. Bar on **L** = 100µm. CBD = corticobasal degeneration.

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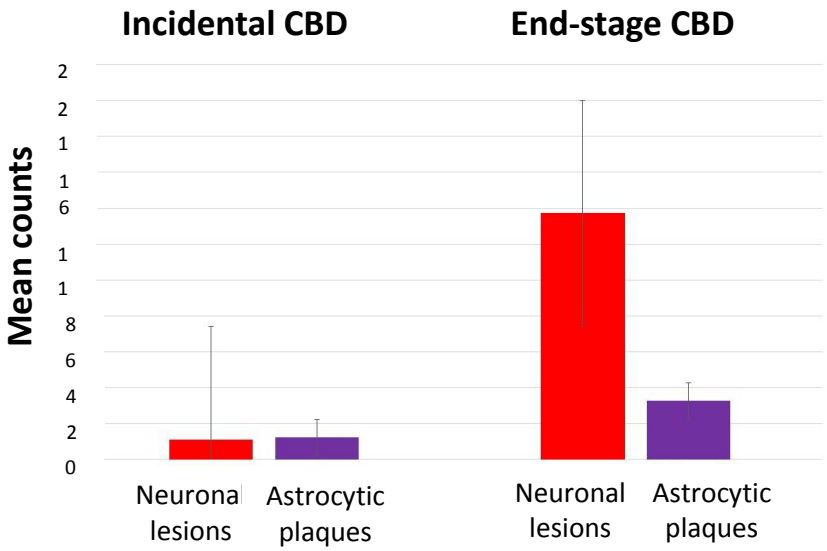

Figure 15 Frequency of neuronal lesions and astrocytic plaques in the cerebral cortex
The neuronal lesions are ~four times greater than astrocytic plaques in end-stage CBD while these lesion types occur with equal frequency in incidental CBD (Error bars represent one standard error of the mean (SEM). CBD = corticobasal degeneration

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8.1 Conclusions of paper 5

The findings of this study showed that, compared with end-stage CBD cases, **1.**) the tau pathology is significantly less severe in the 'incidental' (preclinical) CBD cases, and that **2.**) astrocytic plaques are the predominant tau-positive cellular lesion type in 'incidental' CBD. Our data also indicate that **3.**) the anatomical structures that are first affected by tau deposition in CBD are the basal ganglia and the prefrontal cortex with the posterior frontal cortex, which characteristically shows severe tau pathology in end-stage cases, being only mildly affected in the very early phases of the CBD disease process. **4.**) In the 'incidental' cases the neuronal cell loss is absent or mild in the substantia nigra while this is moderate to severe in the end-stage cases.

9. DISCUSSION OF THE SIGNIFICANCE OF THE FIVE STUDIES

Since the mid-1990s a number of studies, including four of the five neuropathological studies discussed in this thesis, have been carried out in the QSBB, which have resulted in the establishment of the clinical diagnostic criteria of PSP variants such as PSP-P[77] and PSP-PAGF[79] and, as a consequence, in a better understanding of the neuropathological basis of three of the PSP variants[46, 55, 64, 77-79]. The contribution of the QSBB to this area of research and, in particular, its role in the consolidation of the concept of atypical PSP, which by now includes several disease variants (**Table 6**), is widely acknowledged by researchers of other prestigious centres which are active in the field of PSP research[17].

Table 6 Clinicopathological variants of progressive supranuclear palsy

	Basal ganglia/brainstem predominant			**Cortical predominant**				**Cerebellar**
Variant	**PSP-RS**	**PSP-P**	**PSP-PAGF**	**PSP-CBS**	**PSP-FTD**	**PSP-PPA**	**PSP-AOS**	**PSP-C**

PSP = progressive supranuclear palsy; PSP-RS = PSP-Richardson's syndrome; PSP-P = PSP-Parkinsonism; PSP-PAGF = PSP-pure akinesia with gait freezing; PSP-FTD = PSP-frontotemporal dementia; PSP-PPA = PSP-primary progressive aphasia; PSP-AOS = PSP-apraxia of speech; PSP-C = PSP-cerebellar

In the first study, reported in **paper 1**[64], we investigated the nucleus raphe interpositus as the omnipause neurons located in this nucleus had been shown to play an important role in the initiation of saccadic eye movements[12, 31, 32], which are affected in typical PSP, but not in some of the atypical PSP variants[15, 50]. In this study, in which we used stereology for morphometry, we could demonstrate for the first time that the nucleus raphe interpositus is severely affected by severe neuronal cell loss and NFT formation in PSP and that the involvement of this nucleus is significantly more severe in typical PSP than in atypical PSP defined by the absence of SGP. The significance of these findings is at least twofold: 1.) as the integrity of the omnipause neurons is a precondition of normal saccades[12, 31], findings of this study indicate that, in addition to degeneration of a number of other brainstem nuclei with a role in the organisation of saccadic eye movements[40, 53], depletion of and NFT formation in the glycinergic omnipause neurons contribute to the eye movement disorder in PSP and 2.) the PSP variant without SGP can be reliably differentiated

from typical PSP with SGP when appropriate quantitative neuropathological methods, based on stereological principles, are employed.

The major aim of our second study (**paper 2**[55]) was to identify molecular markers that can help to differentiate atypical PSP from the classical form of the disease. In this study, we employed biochemical methods and extracted soluble and insoluble fractions of the tau protein from fresh frozen brain tissue, which we analysed with western blotting. Genetic testing for determining the H1 and H2 *MAPT* haplotypes was also performed. Our findings indicated that the H1/H1 PSP susceptibility genotype and the characteristic PSP-tau doublet electrophoretic migration pattern are prominently associated with typical PSP while the atypical cases, which clinically were more heterogeneous than those studied in **paper 1**, were less often associated with the H1/H1 genotype. Furthermore, the western blot patterns of PSP-tau indicated that in the atypical cases PSP-tau was often enriched in 3R-tau isoforms, which together with 4R-tau isoforms are found in the NFTs in Alzheimer's disease[25] (**Figure 4**). A subsequent study from our brain bank, in which the clinical features of PSP-P were precisely defined, PSP-tau was also biochemically investigated in both PSP-P and PSP-RS cases[77]. This latter study together with that from the large US PSP Brain Bank at the Mayo Clinic[52] not only confirmed that insoluble tau is not exclusively composed of 4R-tau in PSP, but also that in atypical cases, co-existence of Alzheimer's disease-type pathology and apolipoprotein E $\varepsilon 4$ carrier status can influence the tau isoform composition of disease-associated tau in PSP with atypical cases having increased amounts of 3R-tau.

The greatest strength of our first two studies reported in **paper 1** and **paper 2** is that using appropriate neuropathological approaches and molecular markers, we could differentiate atypical PSP from typical PSP thus underpinning the concept that PSP is heterogeneous condition with several disease variants.

Our third neuropathological study (reported in **paper 3**[78]) followed the investigation by Williams *et al*.[77] from the QSBB, which identified the clinical diagnostic criteria of the PSP-P variant[77] in which disease duration is longer and parkinsonism dominates the early clinical picture. Furthermore, Williams *et al*. also firmly established that the most common pathology underlying PAGF is PSP[79]. In our follow-up neuropathological study, the findings

of which were published in **paper 3**[78], we used an unbiased approach for morphometry and performed an extensive quantitative analysis of the tau pathology in typical PSP (PSP-RS), PSP-P and PSP-PAGF. We could demonstrate that there are major quantitative differences in the tau-burden between PSP-RS and the two atypical disease variants in that the deposition of the disease-associated, hyperphosphorylated tau is significantly more severe in PSP-RS than in PSP-P and PSP-PAGF. Furthermore, in both atypical variants the tau pathology is more restricted topographically than in PSP-RS. These findings 1.) contributed to the consolidation of the concept of PSP-P, which is now widely accepted as the most common atypical PSP variant[17, 19, 38] and they also indicate that 2.) there is a close association between clinical phenotype and the severity as well as topographical distribution of the tau pathology.

In our fourth study (reported in **paper 4)**[46] we aimed to understand the neuropathological basis of the PSP-CBS variant. Using unbiased morphometry we could confirm findings of two previous studies[6, 74], which indicated that the cortical tau pathology is significantly increased in PSP-CBS. In addition, we could also demonstrate that, compared with typical PSP controls, the basal ganglia tau-load is significantly reduced in the PSP-CBS cases, which explains why the 'total tau-load' is not different in PSP-RS and PSP-CBS. It is of interest that a similar shift of the tau-load from the basal ganglia to the cerebral cortices has been documented in other cortical PSP variants, including PSP-frontotemporal dementia (PSP-FTD)[7] and PSP-primary progressive aphasia (PSP-PPA)/PSP-apraxia of speech (PSP-AOS)[39]. These observations and the findings reported in **paper 3**[78], strongly support the notion that, as in Alzheimer's disease[8, 17], in PSP also there is a correlation between the severity as well as anatomical distribution of the tau pathology and the clinical phenotype.

The initial major aims of our long-term, still on-going study on CBD were based on the hypothesis that after initiation of the CBD-specific tau pathology, it gradually spreads through established cerebral networks and, by the time disease process reaches the end-stage phase, the tau pathology becomes severe and topographically extensive. Therefore, we wished to understand: 1.) where the tau pathology may start and 2.) which anatomical structures are affected by the tau pathology in the subsequent phases of disease progression in CBD. Analysis of three 'incidental' (clinically asymptomatic or preclinical) cases with CBD

pathology (**paper 5**[47]), which became available from the large archives of CBD cases stored in the QSBB and through collaboration with other UK, European and US centres, allowed us, at least in part, to answer these questions. We showed for the first time that: 1.) 'incidental' CBD cases have significantly less tau pathology than CBD cases with end-stage pathology, which were used as controls, 2.) compared with end-stage cases filamentous astrocytic tau inclusions (astrocytic plaques) are more prominent in 'incidental' CBD than in end-stage CBD cases and that 3.) while in the end-stage cases the posterior frontal cortex and parietal cortex are characteristically severely affected by tau deposition, in the 'incidental' cases the prefrontal cortex was significantly more severely affected than the posterior frontal cortex and the parietal cortex, indicating that involvement of the prefrontal cortex precedes that of the posterior frontal cortex. Finally, 4.) the relatively greater tau burden in the prefrontal cortex and in the striatum as well as in the subthalamic nucleus in the 'incidental' cases, suggests that the striatal afferent connection to the dorsolateral prefrontal cortex and basal ganglia circuitry are likely to be the earliest neural network connections that are affected by the CBD-related tau pathology. The significance of the findings of our fifth study is shown by the scientific commentary that was commissioned by the Editor of Brain[41] and published together with **paper 5** in the same issue of this journal.

Since the publication of **paper 5** in December 2016, we have had the opportunity to study a fourth 'incidental' CBD case, which became available for our ongoing research, courtesy of Professor James Ironside, National CJD Research and Surveillance Unit, Centre for Clinical Brain Sciences, University of Edinburgh (Revesz *et al*. unpublished observations). The significance of this recent observation is that in this case there is no cortical tau deposition and only the basal ganglia structures are affected by characteristic, CBD-specific tau pathology with a predominance of tau-positive astrocytic plaques. This observation has allowed us to outline more accurately the presumed initial steps of disease progression in CBD. Accordingly, 1.) the tau pathology first appears in basal ganglia structures, including the striatum and subthalamic nucleus 2.) whence it spreads to the dorsolateral prefrontal cortex via the striatal-frontal network followed by 3.) further spread of the tau pathology to the posterior frontal cortex using prefrontal-posterior frontal cortico-cortical neuroanatomical connections (**Figure 16**).

(*The last paragraph including Figure 16 is unpublished material, which is included with approval of the Chair of the Board of Graduate Studies*).

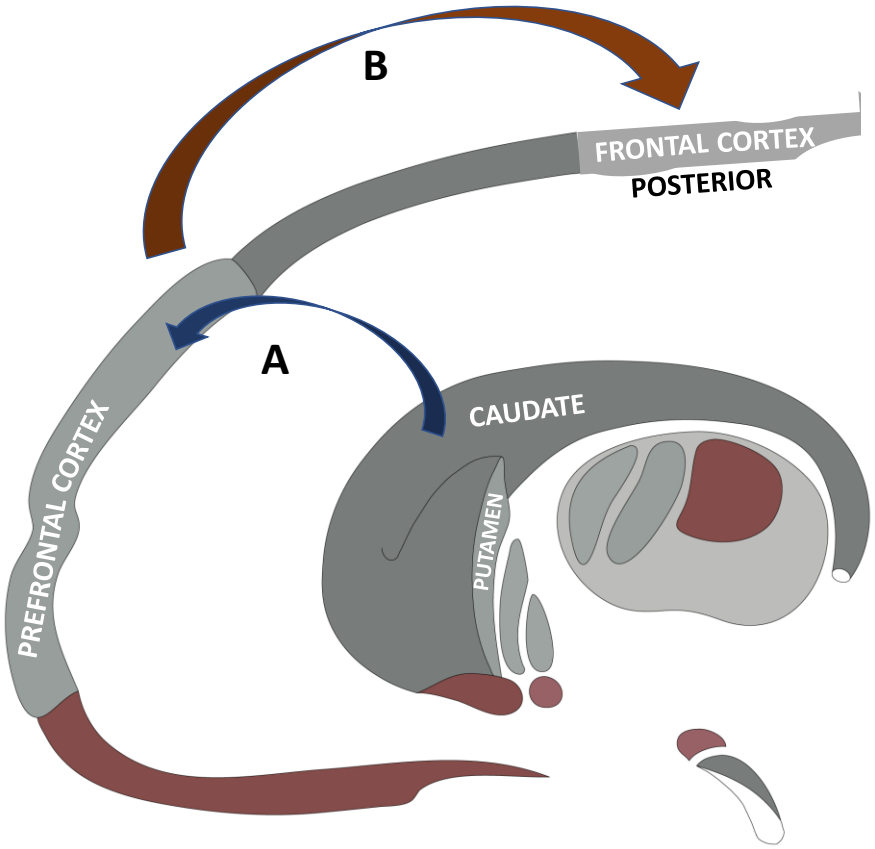

Figure 16 Hypothesis of the anatomical spread of the tau pathology in the initial phases of corticobasal degeneration

A: Striatal-prefrontal connections
B: Prefrontal-posterior frontal connections

Modified after Fig.14.17 in Nieuwenhuys, Voogd and van Huijzen: The Human Central Nervous System. Springer-Verlag, Berlin, 2008

10. FUTURE RESEARCH QUESTIONS

In the last decade, the hypothesis that disease-associated, amyloid-forming fibrillar proteins such as tau replicate important aspects of the behaviour of disease-associated prion protein (PrP^{Sc}) causing prion diseases, has gained considerable support. The notion that fibrillar, disease-associated tau has prion-like properties would indicate 1.) self-amplification of fibrillar tau via the process of 'permissive templating'[29], 2.) cell-to-cell propagation of the aggregation process and that 3.) different tau conformers or 'strains' are able to transmit unique, disease-specific or disease variant-specific pathological information, which could explain the clinicopathological differences that are observed in the different disease variants in both PSP and CBD[1, 13, 14, 24, 56, 71]. Although some experimental data already exist, which raise the possibility that conformationally distinct tau strains may be associated with PSP and CBD[67], future research will be required to show unequivocally that variants of PSP and CBD are also determined by distinct tau strains. One hopes that the continued grant support I have received from the Karin & Sten Mortstedt CBD Solutions AB, Sweden (http://www.cbdsolutions.se) since 2013 and extended in the end of 2017 for further two years, will enable my research group to pursue this line of research and answer some of these fundamentally important questions.

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12. APPENDIX A) SIGNED COPIES OF STATEMENTS OF APPLICANT'S CONTRIBUTIONS^

Papers with Statement of Contribution	Authorship
Paper 1 **The nucleus raphe interpositus in the Steele-Richardson-Olszewski syndrome (progressive supranuclear palsy)** *Brain 1996; 119, 1137-1143* Tamas Revesz conceived the idea of this research. Neuropathological review by SE Daniel and T Revesz, tissue sections were stained by Ms Sangha (pathology technician). T Revesz designed the methods and carried out the morphometry, evaluated the data, carried out the statistical tests and wrote the first draft of the paper. SE Daniel contributed to the final version of the paper.	**Revesz T*** Sangha H Daniel SE

* corresponding author

I agree with the above statement:

Date: 12/4/2018

Professor Andrew J Lees
Head of Department of the Queen Square Brain Bank,
UCL Institute of Neurology at the time when this study was carried out

^ With prior approval of the Chair of the Board of Graduate Studies the declaration about **paper 1** was signed by the head of the department where the research was carried out while the declarations of **papers 2 – 5** were signed by the first authors and authors with significant contributions to these studies.

Paper 2 **Pathological, clinical and genetic heterogeneity in progressive supranuclear palsy** **Brain 2002; 125:969-975** The idea of the project was conceived by T Revesz, SE Daniel, BH Anderton. Neuropathological review of the cases was carried out by T Revesz and SE Daniel. Tau protein extraction and western blot analysis were carried out by T Revesz, G Gibb, D Hanger (T Revesz was supervised by G Gibb and BH Anderton). Data evaluation by D Hanger, Dr Gibb, T Revesz with advice from BH Anderton. Genetic analysis: HR Morris under the supervision of NW Wood. C Strand and T Lashley carried tau immunohistochemistry. The manuscript was written by HR Morris, G Gibb and T Revesz with input from the other co-authors.	Morris HR Gibb G Katzenschlager Wood NW Hanger DP Strand C Lahley T Daniel SE Lees AJ Anderton BH Revesz T*

* corresponding author

I agree with the above statement:

Brian Anderton

Professor Brian Anderton
KCL

Date: 11/1/2018

Professor Huw Morris
UCL

Date: 08/03/2018

Paper 3 **Pathological tau burden and distribution distinguishes progressive supranuclear palsy-parkinsonism from Richardson's syndrome** **Brain 2007; 130:1566-1576** The idea of this neuropathological project was conceived by DR Williams, T Revesz, AJ Lees. Neuropathological review of the cases was carried out by T Revesz and JL Holton. The morphometry was designed by DR Williams and T Revesz as co-supervisor of DR Williams's PhD project. DR Williams carried out the data acquisition. Quality control: T Revesz. Data evaluation by statistical methods by DR Williams, discussing the results with T Revesz. The first draft of the manuscript was written by DR Williams. T Revesz, J Holton and AJ Lees helped with the final, published version of the manuscript.	Williams DR Holton JL Strand C Pittman A de Silva R Lees AJ Revesz T*

* corresponding author

I agree with the above statement:

Professor Andrew J Lees

Date: 25 January 2018

A/Prof. David Williams

Date: 28 February 2018

Paper 4 **Characteristics of progressive supranuclear palsy presenting with corticobasal syndrome: a cortical variant** **Neuropathol Appl Neurobiol 2014; 40:149–163** This neuropathological project was conceived and the methodology designed by T Revesz as the neuropathologist co-supervisor of H Ling's PhD project. Review of clinical aspects of the cases: AJ Lees, H Ling. Neuropathological review: T Revesz. Morphometry and data analysis, including statistics: H Ling. Quality control: T Revesz. Western blotting for tau: R de Silva. The first draft of the manuscript was prepared by H Ling. T Revesz, AJ Lees and JL Holton contributed to the final, accepted manuscript.	Ling H De Silva R Massey LA Courtney R Hondhamuni G Bajaj N Lowe J Holton JL Lees AJ Revesz T*

* corresponding author

I agree with the above statement:

Professor Andrew J Lees

Date: 25/1/18

Dr Helen Ling

Date: 25/1/18

Paper 5 **Astrogliopathy predominates the earliest stage of corticobasal degeneration pathology** **Brain 2016; 139; 3237–3252** This is part of a large, still ongoing project supported by a grant (PI: T Revesz). Design of project: T Revesz. Neuropathological review: T Revesz, JL Holton and H Ling. H Ling was responsible for carrying out the morphometry and data analysis including statistics under the supervision of T Revesz. Regular quality control: T Revesz. Tau immunohistochemistry, digitising stained slides, arranging automated image analysis: K Davey. Genetics: KY Mok under the supervision of J Hardy. GG Kovacs and Vonsattel contributed by providing one case each. HR Morris and TT Warner gave advice on clinical aspects. With advice/help from T Revesz, H Ling prepared the first draft and the final form of the manuscript.	Ling H Kovacs GG Vonsattel JPG Davey K Mok KY Hardy J Morris HR Warner TT Holton JL Revesz T*

**corresponding author*

Dr Helen Ling

Date: 22-2-2018

Professor Janice L. Holton

Date: 10.3.18

13. APPENDIX B) FULL PUBLICATION LIST

H-index: 73 (ISI), 84 (Google Scholar); ResearcherID: A-8732-2010

A. Peer-reviewed publications

A.1 Papers submitted for consideration for a PhD by Published Work

1. **Revesz T**, Sangha H, Daniel SE. The nucleus raphe interpositus in the Steele-Richardson-Olszewski syndrome (progressive supranuclear palsy). **Brain** 1996;119:1137-43.
2. Morris HR, Gibb G, Katzenschlager R, Wood NW, Hanger DP, Strand C, Lashley T, Daniel SE, Lees AJ, Anderton BH, **Revesz T**. Pathological, clinical and genetic heterogeneity in progressive supranuclear palsy. **Brain** 2002;125:969-75.
3. Williams DR, Holton JL, Strand C, Pittman A, de Silva R, Lees AJ, **Revesz T**. Pathological tau burden and distribution distinguishes progressive supranuclear palsy-parkinsonism from Richardson's syndrome. **Brain** 2007;130:1566-76.
4. Ling H, de Silva R, Massey LA, Courtney R, Hondhamuni G, Bajaj N, Lowe J, Holton JL, Lees A, **Revesz T**. Characteristics of progressive supranuclear palsy presenting with corticobasal syndrome: a cortical variant. **Neuropathol Appl Neurobiol** 2014 40:149-163.
5. Ling H, Kovacs GG, Vonsattel JP, Davey K, Mok KY, Hardy J, Morris HR, Warner TT, Holton JL, **Revesz T**. Astrogliopathy predominates the earliest stage of corticobasal degeneration pathology. **Brain** 2016;139:3237-3252.

A.2 Other peer-reviewed papers related to Parkinson's disease and atypical parkinsonism

A.2.1 Parkinson's disease (including genetic forms) and dementia with Lewy bodies

6. Guerreiro R, Ross OA, Kun-Rodrigues C, Hernandez DG, Orme T, Eicher JD, Shepherd CE, Parkkinen L, Darwent L, Heckman MG, Scholz SW, Troncoso JC, Pletnikova O, Ansorge O, Clarimon J, Lleo A, Morenas-Rodriguez E, Clark L, Honig LS, Marder K, Lemstra A, Rogaeva E, St George-Hyslop P, Londos E, Zetterberg H, Barber I, Braae A, Brown K, Morgan K, Troakes C, Al-Sarraj S, Lashley T, Holton J, Compta Y, Van Deerlin V, Serrano GE, Beach TG, Lesage S, Galasko D, Masliah E, Santana I, Pastor P, Diez-Fairen M, Aguilar M, Tienari PJ, Myllykangas L, OinasM, **Revesz T**, Lees A, Boeve BF, Petersen RC, Ferman TJ, Escott-Price V, Graff-Radford N, Cairns NJ, Morris JC, Pickering-Brown S, Mann D, Halliday GM, Hardy J, Trojanowski JQ, Dickson DW, Singleton A, Stone DJ, Bras J. Investigating the genetic architecture of dementia with Lewy bodies: a two-stage genome-wide association study. **Lancet Neurol** 2018 Jan;17(1):64-74. doi:10.1016/S1474-4422(17)30400-3
7. De Pablo-Fernandez E, Tur C, **Revesz T**, Lees AJ, Holton JL, Warner TT. Association of Autonomic Dysfunction With Disease Progression and Survival in Parkinson Disease. **JAMA Neurol** 2017 Aug 1;74(8):970-976. doi: 10.1001/jamaneurol.2017.1125
8. Kun-Rodrigues C, Ross OA, Orme T, Shepherd C, Parkkinen L, ……**Revesz T**, Lees A, Boeve BF, Petersen RC, Ferman TJ, Escott-Price V, Graff-Radford N, Cairns NJ, Morris JC, Stone DJ, Pickering-Brown S, Mann D, Dickson DW, Halliday GM, Singleton A, Guerreiro R, Bras J. Analysis of C9orf72 repeat expansions in a large international cohort of dementia with Lewy bodies. **Neurobiol Aging** 2017 Jan;49:214.e13-214.e15.

9. Ling H, Kearney S, Yip HL, Silveira-Moriyama L, **Revesz** T, Holton JL, Strand C, Davey K, Mok KY, Polke JM, Lees AJ. Parkinson's disease without nigral degeneration: a pathological correlate of scans without evidence of dopaminergic deficit (SWEDD)? **J Neurol Neurosurg Psychiatry** 2016;87:633-41.

10. Massey LA, Miranda MA, Al-Helli O, Parkes HG, Thornton JS, So PW, White MJ, Mancini L, Strand C, Holton J, Lees AJ, **Revesz T**, Yousry TA. 9.4 T MR microscopy of the substantia nigra with pathological validation in controls and disease. **Neuroimage Clin** 2016 Nov 17;13:154-163.

11. Guerreiro R, Escott-Price V, Darwent L, Parkkinen L, Ansorge O, Hernandez DG, Nalls MA, Clark L, Honig L, Marder K, van der Flier W, Holstege H, Louwersheimer E, Lemstra A, Scheltens P, Rogaeva E, St George-Hyslop P, Londos E, Zetterberg H, Ortega-Cubero S, Pastor P, Ferman TJ, Graff-Radford NR, Ross OA, Barber I, Braae A, Brown K, Morgan K, Maetzler W, Berg D, Troakes C, Al-Sarraj S, Lashley T, Compta Y, **Revesz T**, Lees A, Cairns NJ, Halliday GM, Mann D, Pickering-Brown S, Powell J, Lunnon K, Lupton MK; International Parkinson's Disease Genomics Consortium (IPDGC), Dickson D, Hardy J, Singleton A, Bras J. Genome-wide analysis of genetic correlation in dementia with Lewy bodies, Parkinson's and Alzheimer's diseases. **Neurobiol Aging** 2016 Feb;38:214.e7-214.e10. doi:10.1016/j.neurobiolaging.2015.10.028

12. Yarnall AJ, Lashley T, Ling H, Lees AJ, Coleman SY, O'Sullivan SS, Compta Y, **Revesz T**, Burn DJ. Apomorphine: A potential modifier of amyloid deposition in Parkinson's disease? **Mov Disord** 2016 May;31(5):668-75

13. Kiely AP, Ling H, Asi YT, Kara E, Proukakis C, Schapira AH, Morris HR, Roberts HC, Lubbe S, Limousin P, Lewis PA, Lees AJ, Quinn N, Hardy J, Love S, **Revesz T**, Houlden H, Holton JL. Distinct clinical and neuropathological features of G51D SNCA mutation cases compared with SNCA duplication and H50Q mutation. **Mol Neurodegener** 2015 Aug 27;10:41. doi: 10.1186/s13024-015-0038-3

14. Cheshire P, Ayton S, Bertram KL, Ling H, Li A, McLean C, Halliday GM, O'Sullivan SS, **Revesz T**, Finkelstein DI, Storey E, Williams DR. Serotonergic markers in Parkinson's disease and levodopa-induced dyskinesias. **Mov Disord** 2015 May;30(6):796-804. doi: 10.1002/mds.26144

15. De Pablo-Fernandez E, Doherty KM, Holton JL, **Revesz T**, Djamshidian A, Limousin P, Bhatia KP, Warner TT, Lees AJ, Ling H. Concomitant fragile X-associated tremor ataxia syndrome and Parkinson's disease: a clinicopathological report of two cases. **J Neurol Neurosurg Psychiatry** 2015;86:934-6.

16. Swirski M, Miners JS, de Silva R, Lashley T, Ling H, Holton J, **Revesz T**, Love S. Evaluating the relationship between amyloid-β and α-synuclein phosphorylated at Ser129 in dementia with Lewy bodies and Parkinson's disease. **Alzheimers Res Ther** 2014;6(5-8):77.

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A.4 Dementias

A.4.1 Frontotemporal lobar degenerations

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A.4.2 Familial British Dementia, Familial Danish Dementia and other cerebral amyloid diseases

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14. APPENDIX C) COPIES OF THE FIVE PUBLICATIONS INCLUDED IN THE THESIS

Brain (1996), **119**, 1137–1143

The nucleus raphe interpositus in the Steele–Richardson–Olszewski syndrome (progressive supranuclear palsy)

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Summary

As the integrity of the omnipause neurons located in the nucleus raphe interpositus is a prerequisite of normal ocular motility, cell and neurofibrillary tangle densities were determined in 13 Steele–Richardson–Olszewski syndrome (SROS) cases [eight with supranuclear gaze palsy (SGP) and five without] and six controls. Compared with normal controls, cases with SGP were associated with ~50% nerve cell loss ($P < 0.001$), whereas data from cases without SGP were not significantly different ($P = 0.18$). Furthermore, cases with SGP had lower neuronal cell ($P = 0.016$) and higher neurofibrillary tangle densities than those without ($P = 0.011$). These results indicate that the involvement of the omnipause neurons, which are glycinergic, contributes to abnormal eye motility in SROS. Involvement of these glycinergic nerve cells suggests that the degeneration of brainstem structures in SROS affects neurochemically diverse systems; so far other brainstem nuclei concerned with eye motility, and known to be affected in SROS, are cholinergic. The results of this study provide evidence that clinically distinct subgroups of SROS may be differentiated histologically when adequate morphometric techniques are applied.

Keywords: Steele–Richardson–Olszewski syndrome; supranuclear gaze palsy; nucleus raphe interpositus; neuronal cell loss; neurofibrillary tangle

Abbreviations: SGP = supranuclear gaze palsy; SROS = Steele–Richardson–Olszewski syndrome

Introduction

Steele–Richardson–Olszewski syndrome or progressive supranuclear palsy is a late-onset, progressive neurodegenerative condition characterized in its classical form by SGP, axial dystonia, bradykinesia, dysarthria, pseudobulbar palsy and cognitive disturbances (Steele *et al.*, 1964). Although SGP, in association with other signs, is regarded as one of the cardinal clinical features of SROS, it may be absent in a proportion of cases (Dubas *et al.*, 1983). The histological hallmarks of SROS include neuronal cell loss, gliosis and neurofibrillary tangles (Steele *et al.*, 1964). The degenerative changes characteristically affect the basal ganglia, brainstem nuclei and cerebellum, but some cerebral cortical areas are also involved (Takahashi *et al.*, 1989; Hauw *et al.*, 1990).

The ocular motor abnormality observed in SROS classically involves severe impairment of vertical saccades and, usually late in the disease process, of horizontal saccades with relative sparing of the vestibulo-ocular reflexes (Pfaffenbach *et al.*, 1972). Supranuclear gaze palsy in SROS correlates with degeneration of a number of primarily cholinergic brainstem structures which include the rostral interstitial nucleus of the medial longitudinal fascicle, interstitial nucleus of Cajal, superior colliculus and nucleus pontis centralis caudalis (Juncos *et al.*, 1991).

The premotor neural network concerned with saccades lies in the paramedian pontine reticular formation and from a functional viewpoint contains two neuronal cell types: (i) the burst neurons, which are active before saccades, and (ii) pause neurons, which pause before and during eye movements. A subset of pause neurons, which have an on-going firing rate and exert a tonic inhibition on burst neurons, pause before eye movements in all directions and are known as omnipause neurons (Fuchs *et al.*, 1985). In both monkeys and humans the omnipause neurons have been shown to lie in the caudal pontine paramedian tegmentum in an anatomically well-

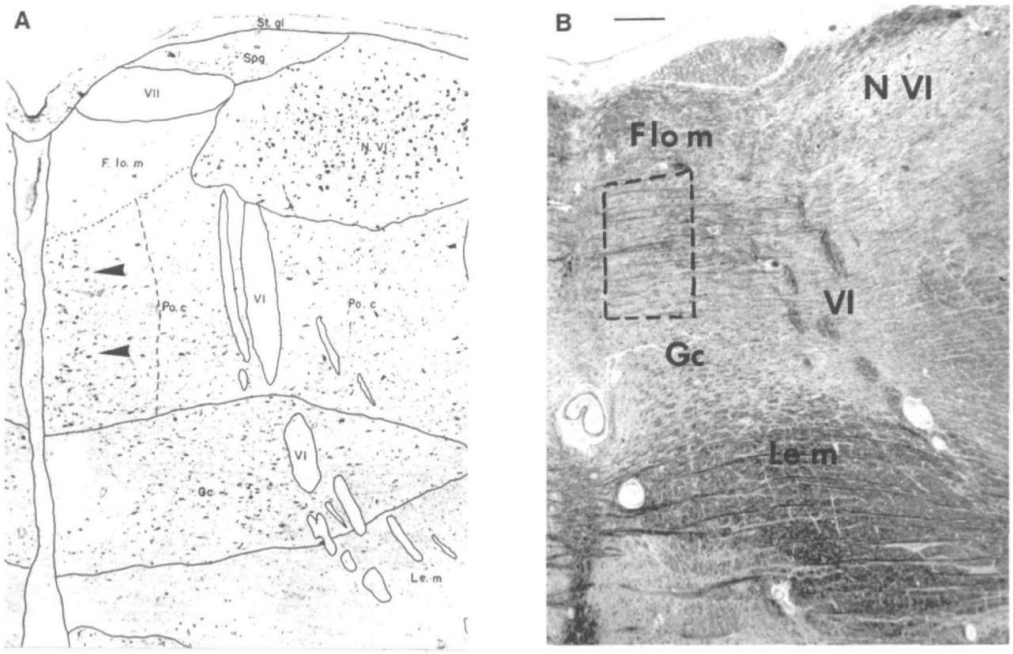

Fig. 1 (**A**) Plate XXIII from the second edition of *Cytoarchitecture of the Human Brain Stem* by Olszewski and Baxter (1982), with permission to reproduce by S. Karger AG, Basel, Switzerland, illustrating the nucleus raphe interpositus in the paramedian tegmentum of the caudal pons (arrowheads). (**B**) Case 3. Cell and neurofibrillary tangle densities were determined in the outlined area of the nucleus raphe interpositus in each case (F lo m = medial longitudinal fascicle, N VI = abducent nucleus, VI = abducent nerve, Gc = gigantocellular nucleus, Le m = medial lemniscus). Luxol fast blue/cresyl violet. Bar = 200 μm.

defined area, which has been designated the nucleus raphe interpositus (Büttner-Ennever *et al.*, 1988). Despite its functional importance the nucleus raphe interpositus has only rarely been examined in pathological conditions (Ridley *et al.*, 1987; Büttner-Ennever *et al.*, 1990).

In this study we examined the nucleus raphe interpositus in 13 SROS cases; eight presented clinically with SGP while the other five did not. The diagnosis was confirmed by full neuropathological examination in all cases.

Material and methods

Thirteen neuropathologically confirmed cases of SROS were selected from the case collections of the Parkinson's Disease Society Brain Tissue Bank and of the Department of Neuropathology, Institute of Neurology, London. Nine of these cases have been reported in detail elsewhere (Daniel *et al.*, 1995). Supranuclear gaze palsy was documented in life in eight of the patients, while it was absent in the remainder. Brains from six age-matched individuals who died of non-neurological disease were used as controls. All the brains used for this study had been immersed in 10% formalin at post-mortem and were then transported to the Brain Tissue Bank or the Department of Neuropathology. The neuropathological examination was carried out 6 weeks later and the tissue blocks were processed using standard techniques.

For this study the paraffin blocks of the lower pons containing the sixth nerve nucleus and the area of the nucleus raphe interpositus were cut serially in 16 μm thick tissue sections. Initially every 10th section was stained with haematoxylin and eosin and the first section containing both the sixth nerve nucleus and its rootlets was identified under a dissecting microscope. Then neighbouring sections were sequentially stained in groups of four, one with luxol fast blue/cresyl violet, one with modified Bielschowsky's silver impregnation for axons, one with antibodies to tau (Sigma, 1:1400) and one with glial fibrillary acidic protein (Dako, 1:400).

Anatomical definitions

As suggested by Büttner-Ennever *et al.* (1988), the presence of the sixth nerve nucleus and its rootlets are important anatomical landmarks of the nucleus raphe interpositus. The area of the nucleus raphe interpositus in the human brainstem

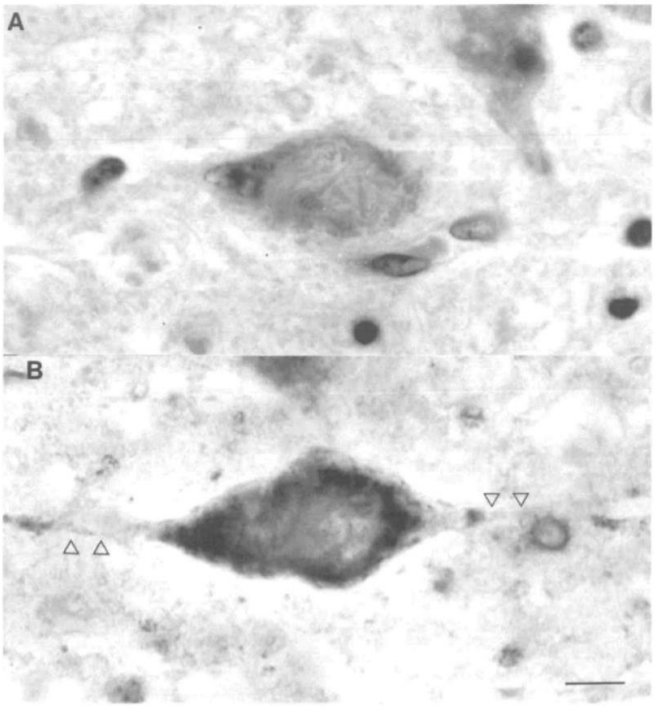

Fig. 2 (**A**) Case 8. Neurofibrillary tangle-bearing fusiform nerve cell in the nucleus raphe interpositus. Haematoxylin and eosin. Bar = 5 μm. (**B**) A neuron with tau-positive neurofibrillary tangle. The open arrowheads point to the horizontally orientated dendrites. Tau immunohistochemistry. Bar = 5 μm.

is outlined, but not named by Olszewski and Baxter (1982). Dorsally, the nucleus is surrounded by the medial longitudinal fascicle and the sixth nerve nucleus, ventrally by the nucleus gigantocellularis, while the lateral border ends before the rootlets of the sixth nerve cross the tegmentum (Fig. 1). The nucleus raphe interpositus comprises omnipause neurons in a linear arrangement on either side of the midline in the dorsal part of the tegmentum. Characteristically each fusiform nerve cell soma and its well-developed dendrites are horizontally orientated (Fig. 2) (Büttner-Ennever *et al.*, 1988; Horn *et al.*, 1994).

Morphometric studies

For cell counts, coded slides were used and the principle of the optical dissector was applied (Gundersen *et al.*, 1988*a*). For the purpose of morphometry a ×100 oil-immersion objective with a high numerical aperture (1.32), providing a depth of field of 0.24 μm (Williams and Rakic, 1988), was used on a Zeiss research microscope. The microscope was fitted with a length gauge (Heidenhain, MT12) and an electronic display unit (Heidenhain, VRZ 405) for monitoring the movements of the stage in the z (focusing) axis. An unbiased grid, calibrated at 0.07×0.07 mm, was projected into the microscopic field via a Zeiss drawing tube fitted to the microscope. A 10 μm high counting box was used and counting was carried out in every third microscopic field. In the area of the nucleus raphe interpositus, nucleolated nerve cells were counted if they were either situated fully inside the counting box or their nuclei did not cross any of the forbidden planes (Gundersen *et al.*, 1988*a*). As the total volume of the nucleus raphe interpositus was unknown, neuronal cell densities were calculated. As cell densities may be distorted by tissue shrinkage due to the disease process, a correction factor was calculated for all three groups of cases on the assumption that tissue shrinkage resulting from the disease process was proportional to the decrease in the height of the nucleus raphe interpositus. To estimate this decrease, a ratio (R) was calculated for each case comparing the height of the nucleus raphe interpositus (h_{NRI}) with the whole height of the pons (h_{pons}):

$$R = \frac{h_{NRI}}{h_{pons}}$$

Measurements were carried out on an image analyser (Colourmorph, Perceptive Instruments, UK). The mean ratios (SD; range) in the SROS groups with SGP (R_{SROS1}) and

Table 1 *Basic clinical data of 13 patients with SROS*

Case no.	Sex	Age at death (years)	Disease duration (years)	Gaze palsy
1	M	67	10	+
2	F	64	5	+
3	M	72	4	+
4	M	69	2	+
5	F	57	5	+
6	M	71	9.5	+
7	M	74	6	+
8	M	65	6	+
9	M	75	4	–
10	M	82	7	–
11	M	76	4	–
12	M	86	9	–
13	F	88	9	–

without SGP (R_{sros2}), and in the control group (R_{contr}) were 0.1354 (0.03; 0.0889–0.1930), 0.1859 (0.048; 0.1363–0.2567), and 0.1886 (0.023; 0.1654–0.2266), respectively. The difference between the SROS group with SGP and the control group was statistically significant (P = 0.003, Student's two-tailed t test, significant at the 1% level allowing for multiple comparisons). The mean ratios were used to calculate a correction factor for each group (x_{contr} for the control group; x_{sros1} for SROS with SGP and x_{sros2} for SROS without SGP) and normalized so that x_{contr} = 1, on the basis that no disease process leading to tissue shrinkage was present in the control cases. Thus:

$$x_{sros1} = \frac{R_{sros1}}{R_{contr}} \text{ and } x_{sros2} = \frac{R_{sros2}}{R_{contr}}$$

In this study the correction factors were x_{sros1} = 0.72 and x_{sros2} = 0.98. These were used to determine an adjusted neuronal cell density (CD) for each case:

$$\text{adjusted CD} = \text{CD} \times \text{correction factor}$$

The density of neurofibrillary tangles (number per mm^2 of section) was also determined in the area of the nucleus raphe interpositus, by using neighbouring tissue sections stained with haematoxylin and eosin, modified Bielschowsky's and anti-tau antibody. Neurofibrillary tangles were counted on all three preparations with a ×40 objective and the highest number per section found was selected for each case.

For statistical analysis, means and medians were contrasted by using the two-tailed Student's t test and the two-tailed Mann–Whitney U test as appropriate. In addition regression statistics were also applied.

Results

The main clinical findings are summarized in Table 1.

The mean ages (SD; range) of the SROS patients and control individuals were 72.7 years (8.9; 57–88 years) and 78.8 years (5.3; 72–85 years), respectively (P = 0.14, two-tailed Student's t test). The mean age (SD; range) was 67.3 years (5.4; 57–74 years) in the SROS group with SGP and 81.4 years (5.8; 75–88 years) in the group without SGP (P = 0.001, two-tailed Student's t test, significant at the 1% level allowing for multiple comparisons). At death, the SROS patients with SGP were also younger than the control individuals (P = 0.002, two-tailed Student's t test, significant at the 1% level allowing for multiple comparisons), but those without SGP were not younger than the controls (P = 0.46, two-tailed Student's t test). There was no difference in the duration of disease between the two groups of SROS cases (P = 0.662, two-tailed Student's t test), the mean disease duration (SD; range) being 5.9 years (2.6; 2–10 years) in the SROS group with SGP and 6.6 years (2.5; 4–9 years) in the group without SGP.

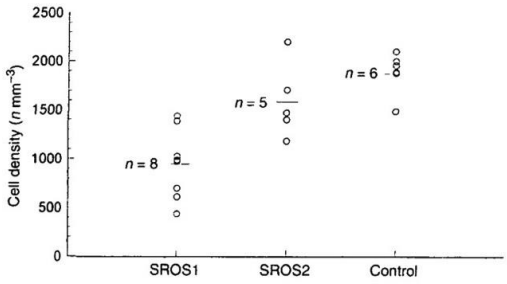

Fig. 3 Adjusted nerve cell densities in the nucleus raphe interpositus. SROS1 = cases with SGP; SROS2 = cases without SGP.

Microscopical examination of the nucleus raphe interpositus

The tegmentum of the lower pons appeared shrunken in many of the SROS cases and particularly in those with SGP. Increased astrocytosis was demonstrated with the glial fibrillary acidic protein preparation in all SROS cases with SGP and in four cases without SGP. Many nerve cells in the nucleus raphe interpositus appeared to contain neurofibrillary tangles (Fig. 2) and there was only a single case without the clinical history of SGP in which this abnormality was absent.

Morphometry

The uncorrected mean neuronal cell density per mm^3 (SD; range) was 1314.9 (492; 609.2–1998) in the SROS group with SGP (n = 8), 1627.4 (396.3; 1208.4–2248.4) in the group without SGP (n = 5) and 1888 (211; 1488–2102) in the six control cases. The difference between the neuronal cell densities of the SROS group with SGP and the control group was statistically significant (P = 0.014, two-tailed Student's t test, significant at the 5% level allowing for multiple comparisons).

The mean adjusted neuronal cell density per mm^3 (SD; range) was 946.8 (354.3; 438.6–1438.6) in the SROS group

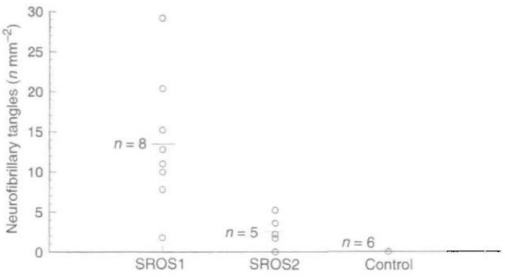

Fig. 4 Neurofibrillary tangle densities in the nucleus raphe interpositus. SROS1 = cases with SGP; SROS2 = cases without SGP.

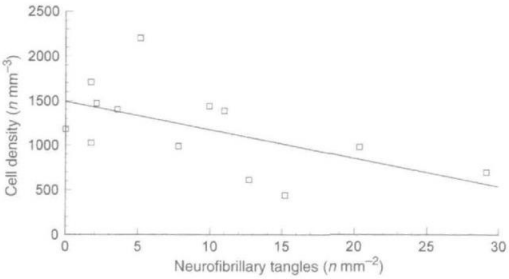

Fig. 5 The relationship between nerve cell density and neurofibrillary tangle density in the nucleus raphe interpositus of SROS cases; note the inverse relationship (r = –0.5647, P = 0.04).

with SGP, 1594.8 (388.4; 1184.2–2203.4) in the group without SGP and 1888 (211; 1488–2102) in the controls (Fig. 3). By using adjusted neuronal cell densities we found a 49.8% cell loss in the SROS group with SGP compared with normal controls ($P < 0.001$, two-tailed Student's t test, significant at the 1% level allowing for multiple comparisons). Furthermore, a significant difference was found in adjusted neuronal cell densities between the two SROS groups (P = 0.016, two-tailed Student's t test, significant at the 5% level allowing for multiple comparisons). However, the 15.5% cell loss found in the SROS group without SGP, compared with controls did not reach statistical significance (P = 0.18, two-tailed Student's t test).

The mean density (SD; range) of neurofibrillary tangles (number per mm^2) in the SROS groups with and without SGP was 13.5 (8.3; 1.8–29.2) and 2.5 (1.9; 0–5.2), respectively (Fig. 4), and the difference between the two groups was statistically significant (P = 0.011, two-tailed Mann–Whitney U test). There tended to be an inverse relationship between the adjusted neuronal cell and neurofibrillary tangle densities (Fig. 5); cases with lower cell densities tended to be associated with higher neurofibrillary tangle values (r = –0.5267, P = 0.04).

Discussion

In this study we have shown that the nucleus raphe interpositus is severely affected in SROS; the mean cell density in patients with SGP was ~ 50% of that in controls ($P <$ 0.001). Furthermore, cases with SGP had a greater cell loss (P = 0.016) and larger mean neurofibrillary tangle density (P = 0.011) than those without eye movement abnormality. An inverse relationship between cell and neurofibrillary tangle densities in the nucleus raphe interpositus has also been established (P = 0.04).

Neurofibrillary tangles, neuronal cell loss and gliosis occurring in certain subcortical brainstem and cerebellar structures are the classical pathological hallmarks of SROS (Steele *et al.*, 1964; Jellinger and Bancher, 1992; Lantos, 1994). It has been suggested that the development of neurofibrillary tangles precedes neuronal degeneration and loss (Seitelberger, 1969), and areas with the most severe nerve cell depletion usually show the largest number of tangles in this condition (Lantos, 1994). The precise cellular mechanisms which initiate neurofibrillary tangle formation and finally cell death in SROS are not clear. However, the existence of an intimate relationship between tangle formation and cell death has been shown in Alzheimer's disease, where the occurrence of neurofibrillary tangles, in general, appears to correlate with the number of nerve cell nuclei with DNA fragmentation, which is an important indicator of nerve cell death through apoptosis (Lassmann *et al.*, 1995).

In typical SROS cases degeneration of midbrain cholinergic structures (including the rostral interstitial nucleus of the medial longitudinal fascicle, nucleus interstitial of Cajal and superior colliculus) appears to be crucial for the development of SGP in the vertical plane (Juncos *et al.*, 1991). The cholinergic nucleus pontis centralis caudalis, which is coextensive with the caudal portion of the paramedian pontine reticular formation, is also severely affected with a loss of up to 60% of its neurons (Malessa *et al.*, 1991). Degeneration of the nucleus pontis centralis caudalis may perhaps be related to abnormal horizontal saccades, which usually appear late in SROS (Troost and Daroff, 1977). The results of our study have shown that, in addition to cholinergic midbrain and pontine structures, omnipause neurons in the nucleus raphe interpositus are also affected in SROS. Omnipause neurons use glycine as a neurotransmitter and receive numerous contacts from GABAergic, glycinergic and glutaminergic afferents; these neurons act as a gating mechanism and exert tonic inhibition both on the horizontal saccadic burst neurons in the paramedian pontine reticular formation and on the vertical saccadic burst neurons in the rostral interstitial nucleus of the medial longitudinal fascicle (Büttner-Ennever *et al.*, 1988; Nakao *et al.*, 1989; Horn *et al.*, 1994). The morphological and functional integrity of omnipause neurons is a prerequisite to normal saccades (Büttner-Ennever *et al.*, 1988; Nakao *et al.*, 1989, 1991; Langer and Kaneko, 1990; Horn *et al.*, 1994). Our finding that omnipause neurons are severely depleted and affected

by neurofibrillary tangle formation in cases of SROS with SGP, and only to a lesser degree in those without SGP, suggests that degeneration of the nucleus raphe interpositus contributes to the abnormal eye movements in this condition. Furthermore, our observation that the glycinergic omnipause neurons are severely depleted in the paramedian pontine tegmentum in SROS with SGP, combined with the above-mentioned studies of degeneration of cholinergic brainstem structures, indicates that the disease process is both biochemically and regionally diverse.

In recent years it has been confirmed that SROS is clinically a rather heterogeneous condition; atypical cases presenting without SGP (Dubas *et al.*, 1983), with severe dementia (Davis *et al.*, 1985) or pure akinesia (Matsuo *et al.*, 1991) and also a familial form of the disease (Brown *et al.*, 1993) have all been documented. It is possible that the differences in clinical presentation may delineate distinct subgroups in SROS; patients presenting without SGP appear to be older at the disease onset, have a longer disease duration and are more often females than males (de Bruin and Lees, 1992; Daniel *et al.*, 1995). Histological heterogeneity of SROS has also been described here and a new classification recommended, which distinguishes three subgroups: (i) typical (type 1) cases with histological features corresponding to the original description; (ii) atypical (type 2) cases in which either the severity or the distribution of changes deviates from those in typical cases; and (iii) combined (type 3) cases in which the typical histological picture of SROS is associated with features of another neurodegenerative condition (Hauw *et al.*, 1994; Lantos, 1994). Morphologically, however, such subtypes are difficult to discriminate particularly when they rely on qualitative assessment alone (Gearing *et al.*, 1994; Daniel *et al.*, 1995). Other attempts at histological subclassification have been made from a clinical perspective. Investigations in one such study of 17 SROS cases failed to show any histological differences when SGP was used as discriminating criterion (Daniel *et al.*, 1995). We report the novel finding that if careful morphometric evaluation is applied to these cases, a histological distinction can be made. Furthermore, the results of this study extend our understanding of the morphological basis of eye movement abnormality in SROS.

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Pathological, clinical and genetic heterogeneity in progressive supranuclear palsy

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Summary

We have identified two groups of patients with clinically typical and atypical, pathologically diagnosed progressive supranuclear palsy (PSP), and investigated their genetic and molecular pathological characteristics. Those with clinically typical PSP are more likely to have the PSP susceptibility genotype and to have the deposition of PSP-type hyperphosphorylated tau protein. The clinically atypical PSP group contains a number of different clinical syndromes, including an L-dopa unresponsive bradykinetic syndrome and a clinical syndrome closely resembling idiopathic Parkinson's disease. The clinically atypical PSP group are less likely to have the PSP susceptibility genotype and often have the deposition of Alzheimer's disease paired helical filament type hyperphosphorylated tau. This study suggests that the tau PSP susceptibility genotype is most strongly associated with clinically typical PSP. Neurofibrillary tangle parkinsonian disorders, which pathologically resemble PSP but involve the deposition of Alzheimer's disease-type tau often without involvement of the tau susceptibility genotype, need to be distinguished for diagnostic and research purposes.

Keywords: genotype; immunoblotting; neurofibrillary tangle; progressive supranuclear palsy; tau

Abbreviations: FTDP-17 = frontotemporal dementia with parkinsonism linked to chromosome 17; NFT = neurofibrillary tangle; PHF = paired helical filament; PSP = progressive supranuclear palsy

Introduction

The characteristic pathological and clinical features of progressive supranuclear palsy (PSP) were first delineated by Steele, Richardson and Olszewski in the early 1960s (Steele *et al.*, 1964). Like Alzheimer's disease, the pathology of PSP involves the deposition of abnormally hyperphosphorylated tau containing neurofibrillary tangles (NFTs), but the pathological topography and absence of amyloid plaque formation distinguishes the two disorders (Hauw *et al.*, 1994). PSP presents with progressive gait instability with backwards falls, signs of parkinsonism, a vertical supranuclear gaze palsy, frontal dysfunction, axial rigidity and a pseudo-bulbar palsy (Litvan *et al.*, 1996). Subcortical NFT formation with a predilection for the globus pallidus, subthalamic nucleus, substantia nigra and reticular formation of the midbrain and pons is found on pathological examination. The destruction of the midbrain reticular formation includes damage to nuclei thought to be important in the supranuclear control of vertical gaze, and the widespread damage to basal ganglia output pathways may explain L-dopa unresponsiveness and axial parkinsonism (Hauw *et al.*, 1994). The pathologically based clinical features of PSP have been developed into operational research criteria for the diagnosis of definite, probable or possible PSP (Litvan *et al.*, 1996).

A number of authors have reported different clinical syndromes in patients with pathologically diagnosed PSP. Davis and colleagues reported four patients with atypical presentations of PSP (Davis *et al.*, 1985). Two of these patients had prominent cortical dysfunction and two had normal eye movements. A further larger series of patients with pathologically diagnosed PSP and normal eye movements was reported in 1995, raising the possibility of a 'clinically atypical' PSP subgroup, with a more benign

prognosis (Daniel *et al.*, 1995). Further detailed analysis of the neuropathology of this group has demonstrated that the patients without a supranuclear gaze palsy have less damage to the omnipause neurones located in the pontine nucleus raphe interpositus (Revesz *et al.*, 1996).

In recent years the molecular pathology of tau deposition in PSP, Alzheimer's disease and related disorders has been studied intensively. In Alzheimer's disease the microtubule-associated protein tau is deposited as abnormally phosphorylated paired helical filaments (PHFs) and forms a major triplet of bands on immuno-electrophoresis at 59, 64 and 69 kDa, with a weaker band at 71 kDa (Hanger *et al.*, 1991; Goedert, 1993; Mulot *et al.*, 1994). Dephosphorylation of Alzheimer's disease tau indicates that it consists of all six isoforms of the alternatively spliced *tau* gene (Goedert *et al.*, 1992). In contrast, in PSP, tau is deposited as a major doublet of hyperphosphorylated tau of 64 and 69 kDa (Flament *et al.*, 1991), predominantly consisting of the four repeat tau protein isoforms, which contain the microtubule binding domain encoded by the alternatively spliced exon 10 (Mailliot *et al.*, 1998; Spillantini and Goedert, 1998). NFTs in PSP ultrastructurally appear as 15–18 nm straight filaments, but filaments with a long periodicity have also been described (Tellez-Nagel and Wisniewski, 1973; Roy *et al.*, 1974; Lee *et al.*, 2001). Thus, PSP and Alzheimer's disease differ in the type of tau protein deposition, in addition to the differences in topography and amyloid deposition. It is unclear whether these tau protein differences reflect the specific cell types/regions involved, or are global characteristics of the diseases.

The importance of tau in PSP was underlined by the identification of a genetic susceptibility to PSP defined by the *tau* H1 haplotype (Conrad *et al.*, 1997; Baker *et al.*, 1999). Although this H1 haplotype accounts for ~70% of control haplotypes it makes up 85–95% of PSP tau haplotypes in clinically diagnosed series (Bennett *et al.*, 1998; Higgins *et al.*, 1998; Oliva *et al.*, 1998; Hoenicka *et al.*, 1999; Morris *et al.*, 1999). The functional consequence of this over-representation of the H1 haplotype in PSP is unknown, although one recent study suggested that it has no major influence on the pathological or biochemical phenotype of PSP (Liu *et al.*, 2001). Furthermore, in rare autosomal dominant FTDP-17 (frontotemporal dementia with parkinsonism linked to chromosome 17) families, pathogenic mutations in *tau* may lead to neurodegeneration (Hutton *et al.*, 1998; Poorkaj *et al.*, 1998; Spillantini *et al.*, 1998), and in some cases the clinical and pathological features of affected family members may closely resemble PSP (Murrell *et al.*, 1997; Delisle *et al.*, 1999; Stanford *et al.*, 2000). This emerging information on the pathology, genetics and pathogenesis of tau-related neurodegeneration has led us to re-examine the features of the 'clinically atypical' PSP series reported in 1995 and compare them with 'clinically typical' cases, in an attempt to further define the nosology of this condition and to provide more evidence for the possible role of the tau H1 haplotype.

Material and methods

Genetic analysis

DNA was extracted from frozen brain using standard methods. The tau haplotype was assigned by analysis of the tau intronic microsatellite polymorphism and the exon 9i single nucleotide polymorphism as described previously (Baker *et al.*, 1999).

Pathological diagnosis

Neuropathologically confirmed cases of PSP in which frozen tissue was available were taken from the Queen Square Brain Bank for Neurological Disorders, at the Institute of Neurology (London, UK). After post-mortem the brains were bisected and one half brain immediately frozen and stored at –70°C, while the other half was immersed in 10% (v/v) neutral formalin. Tissue blocks were processed using standard protocols.

Tau protein analysis

Whenever it was available the globus pallidus was selected for protein analysis (23 cases). In eight cases the pons was also used for additional studies, and in the three cases in which these areas were not available, the putamen was selected. Insoluble tau was extracted using a previously described method (Hanger *et al.*, 1998). In brief, 0.1–0.2 g of brain tissue was hand-homogenized in 50 mM MES (2-[*N*-morpholino]ethanesulfonic acid) buffer, pH 6.5, containing 1 M NaCl, 50 mM imidazole, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 20 mM NaF, 10 mM Na^+ pyrophosphate and 25 mM Naβ-glycerophosphate, and the homogenate was centrifuged at 27 000 *g* for 30 min at 4°C. The supernatant was retained and re-centrifuged at 100 000 *g* for 60 min at 4°C. The pellet of the second centrifugation step was solubilized in Laemmli sample buffer and analysed on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) using a discontinuous buffer system (Laemmli, 1970). Western blotting with the rabbit polyclonal antiserum TP70 (Brion *et al.*, 1993), which recognizes all forms of tau, and with the phosphorylation-dependent monoclonal antibody PHF-1 (a kind gift from P. Davies) was subsequently carried out on the resolved proteins using enhanced chemiluminesence (Amersham Biosciences, Little Chalfont, UK).

Results

The results presented below are summarized in Tables 1 and 2.

Clinical features

Among 26 pathologically diagnosed cases of PSP we identified 15 clinically atypical and 11 clinically typical

Table 1 *Clinical, pathological, genetic and biochemical data*

Case	PSP diagnosis by clinical criteria	Working clinical diagnosis	Pathological features	AD-type pathology	Age at onset (years)	Age at death (years)	Tau haplotype	Area used for western blotting	Tau protein pattern
1	Atypical	PD	PSP and involvement of parietal cortex	PA	70	77	H1H1	Putamen	Doublet
2	Atypical	PD	PSP	PA	74	82	H1H1	Putamen	Non-doublet
3	Atypical	PD	PSP and involvement of mesial temporal cortex	PA	76	91	H1H1	GP/pons	Non-doublet
4	Atypical	PD	PSP and relative sparing of the pallidum	0	53	70	H2H2	GP	Non-doublet
5	Atypical	PD	PSP*	0	72	79	H1H1	GP/pons	Doublet
6	Atypical	AD	PSP and involvement of the frontal cortex	PoAD	77	80	H1H2	GP/pons	Doublet
7	Atypical	PD	PSP	PA	56	77	H1H2	GP	Non-doublet
8	Atypical	PD	PSP and involvement of temporal cortex	PA	56	61	H1H2	GP	Non-doublet
9	Atypical	PEP	PSP and perivascular lymphocytic cuffing	PA	58	68	H1H1	GP	Non-doublet
10	Atypical	PD, Atypical	PSP and involvement of mesial temporal cortex, relative sparing of the pons	PA	77	84	H1H1	GP	Non-doublet
11	Typical	PSP	PSP and involvement of mesial temporal cortex	PA	66	68	H1H1	GP	Doublet
12	Atypical	PD	PSP	0	59	72	H1H1	GP	Doublet
13	Typical	PSP/CBD	PSP and CBD-like features	PA	63	66	H1H1	GP/pons	Non-doublet
14	Atypical	PD	PSP and involvement of mesial temporal cortex, relative sparing of the pons	PA	76	86	H1H1	GP	Non-doublet
15	Typical	PSP	PSP	PoAD	74	79	H1H1	GP	Doublet
16	Typical	PSP	PSP	PA	68	72	H1H1	GP	Doublet
17	Typical	PSP	PSP	0	65	73	H1H1	GP	Non-doublet
18	Typical	PSP	PSP and diffuse cortical involvement	PA	64	72	H1H1	GP	Doublet
19	Typical	PSP	PSP	PA	65	75	H1H1	GP/pons	Non-doublet
20	Typical	PSP	PSP	PA	64	69	H1H1	GP	Doublet
21	Typical	PSP	PSP	0	64	72	H1H1	GP/pons	Doublet
22	Typical	PSP	PSP	PA	67	72	H1H1	Putamen	Doublet
23	Atypical	PSP	PSP*	0	62	69	H1H1	GP	Non-doublet
24	Typical	PSP	PSP	PA	66	67	H1H1	GP/pons	Doublet
25	Atypical	CBD	PSP	0	55	64	H1H1	GP	Doublet
26	Atypical	PD	PSP and cerebrovascular disease	PA	74	85	H1H1	GP/pons	Non-doublet

*Limited tissue available. 0 = no Aβ pathology; AD = Alzheimer's disease; CBD = corticobasal degeneration; GP = globus pallidus; PA = pathological ageing; PD = Parkinson's disease; PEP = post-encephalitic parkinsonism; PoAD = possible Alzheimer's disease; PSP = progressive supranuclear palsy. Case 9 had oculogyric crisis, but met pathological criteria for PSP.

cases. The clinical features were identified on the basis of retrospective notes review and the cases were referred from a variety of sources between 1987 and 1998. In some case records there was incomplete recording of eye movement examination, onset symptoms and onset of balance disturbance. The clinically typical cases all met the Tolosa criteria for PSP with slow vertical saccades or a more severe vertical eye movement disorder, falls/postural instability and additional supportive features (Tolosa *et al*., 1994). Five of these cases did not have falls in the first year following symptom onset, and in one case the presence or absence of falls was not documented; therefore, only five of these clinically typical cases met the more rigorous NINDS criteria for the diagnosis of PSP, which specify falls in the first year of symptoms (Litvan *et al*., 1996). The clinically atypical group had a variety of clinical syndromes. Four clinically atypical cases had idiopathic Parkinson's disease-like presentations with an asymmetrical onset and a good response to L-dopa treatment. Four cases had non-L-dopa-responsive parkinsonism, and three of these four cases were documented to have normal eye movements. Two cases had an eye movement disorder with relatively preserved balance and some response to L-dopa. One case had a corticobasal degeneration syndrome with asymmetrical dystonia and apraxia. The remaining four cases had insufficient clinical documentation for confident clinical diagnosis, but three of these cases had significant postural instability, raising the possibility that they had an unrecognized eye movement disorder and were in fact typical PSP cases.

Pathological features

The distribution of NFTs in the cases identified was consistent with the diagnosis of PSP. Some cases had more prominent cortical involvement, in particular the mesial temporal cortex, and some cases had sparing of one of the areas typically affected in PSP, usually the basis pontis. These atypical features were more common in the clinically atypical groups (pathologically atypical features 7/15 versus 2/11). In order to determine whether concurrent Alzheimer-type pathology could account for the differences in tau deposition, we examined the cases for Alzheimer-type pathology. The

Table 2 *Comparison between clinically atypical and typical cases of PSP*

	n	Mean age at onset (years)	Mean age at death (years)	H1/H1 haplotype (%)	Doublet band formation (%)	Extra hippocampal neuritic plaques (%)
Clinically atypical	15	66.3	76.3	73.3	33.3	26.7
Clinically typical	11	66	71.4	100	72.7	18.2

majority of the cases with Alzheimer-type pathology had mesial temporal NFTs with or without mesial temporal and in some cases also with neocortical, mainly diffuse, plaques. Such cases were defined as showing pathological ageing. In two cases the number of neuritic plaques was such that, using the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) criteria, they met the diagnostic criteria of possible Alzheimer's disease (Mirra *et al.*, 1991). The average age at death of cases with extra-hippocampal plaque formation cases was greater than those without (80 versus 72 years).

Genetic and molecular features

The majority of the clinically typical PSP cases had the normal PSP tau protein electrophoretic pattern (Flament *et al.*, 1991) as compared with about one-third of the clinically atypical cases (73% versus 33%). This pattern of the insoluble tau-enriched protein fraction labelled with TP70 contains the upper two bands of PHF-tau plus a fourth, faint, slowest migrating protein band (Fig. 1, lanes 1 and 2). However, abnormal PSP tau protein profiles could be divided into two types of pattern: the first banding pattern was very similar to PHF-tau in that there were three major protein species that aligned with the three major tau bands in PHF-tau (Fig. 1, lanes 1 and 3), as well as the fourth, faint protein species, which is present in both PHF-tau and PSP-tau. The second abnormal PSP tau protein array demonstrated six to eight protein bands, which migrated similarly to the six recombinant tau isoforms (Fig. 1, lanes 4 and 5). Interestingly, when the normal and abnormal PSP samples were probed with the phosphorylation-dependent antibody PHF-1, they showed a similar tau protein staining pattern (Fig. 1, lanes 6–8), although the relative intensity of staining of the tau bands varied between cases. The fourth uppermost weak band was more readily observed with the PHF-1 antibody (Fig. 1, lanes 6–8). The occurrence of abnormal western blot profiles did not correlate with the presence of Alzheimer-type pathology (9/19 versus 3/7) or of pathologically atypical features (5/9 versus 8/17).

All clinically typical, pathologically typical PSP cases were homozygous for the PSP susceptibility genotype H1H1, compared with 73% of the clinically atypical cases. Conversely, the majority of all cases with the deposition of the normal PSP-type tau possessed the PSP susceptibility genotype (12/13, 92%).

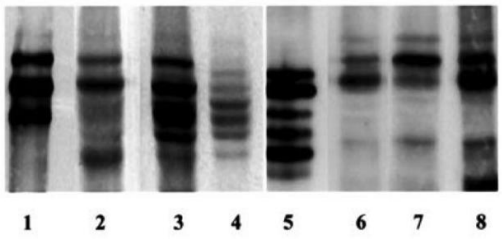

Fig. 1 Insoluble tau electrophoretic patterns stained with antibody TP70 (lanes 1–5) and PHF-1 antibody (lanes 6–8). Lane 1, Alzheimer's disease; lanes 2 and 6, normal PSP pattern; lanes 3 and 7, abnormal PSP pattern 1; lanes 4 and 8, abnormal PSP pattern 2; lane 5, six recombinant tau isoforms.

Discussion

This study suggests that there may be a clinically atypical PSP subgroup in which a classical distribution of PSP pathology occurs, but with the deposition of Alzheimer's disease-type tau protein rather than PSP-type tau. The atypical PSP subgroup, which is defined on the basis of an atypical clinical presentation, is less likely to have the PSP susceptibility genotype and more likely to have an Alzheimer's disease tau deposition pattern. Some of these cases are likely to correspond to the pathologically atypical PSP subgroup defined in the preliminary NINDS criteria for the pathological diagnosis of PSP. The pathologically atypical subgroup was defined by more marked cortical involvement and sparing of some subcortical areas, including the basis pontis (Hauw *et al.*, 1994). The differences in tau protein deposition in this series cannot be explained by regional differences in tau pathology, since the same brain areas were studied, and is not explained by concurrent Alzheimer-type pathology associated with ageing. Previous studies have shown that in PSP cases a PHF-type tau triplet electrophoretic migration pattern may be seen on immunoblots from tissue samples of the mediotemporal region when it is affected by NFT formation related to ageing. In such cases, however, a PSP-type tau doublet pattern is seen when tissue samples from areas other than the mediotemporal region are used for immunoblotting (Vermersch *et al.*, 1994; Schmidt *et al.*, 1996). There are a number of arguments supporting the notion that pathological ageing alone does not

explain the abnormal tau protein patterns seen in our atypical groups. (i) There was no difference in the involvement by senile plaques and Alzheimer's disease-type NFTs between the disease groups. (ii) In this study the globus pallidus and pontine base were used for immunoblotting in all but three cases, as these structures are not usually affected by Alzheimer's disease-type neurofibrillary pathology (Braak and Braak, 1994). The value of this approach is supported by our finding of a normal PSP-type tau doublet pattern on the immunoblots of the two (one clinically typical and one atypical) PSP cases, in which the diagnosis of possible Alzheimer's disease was established, in addition to PSP.

This combination of molecular and topographical pathology in these Caucasian cases is most similar to the pathology seen in the parkinsonism dementia complex of Guam (PDC), the FTDP-17 family with the tau R406W mutation and post-encephalitic parkinsonism (PEP) (Geddes *et al.*, 1993; Reed *et al.*, 1997). In these conditions subcortical deposition of PHF-type tau occurs (Buee-Scherrer *et al.*, 1997; Reed *et al.*, 1997; Perez-Tur *et al.*, 1999). However, in these conditions mesial temporal involvement may be more extensive than is seen in PSP, and this is usually accompanied by clinical amnesia, in PDC and FTDP-17. PDC, PEP and R406W cases do not usually have an idiopathic Parkinson's disease-type clinical presentation. There are three further conditions in which subcortical tau NFT deposition occurs with parkinsonism. These are autosomal recessive parkinsonism linked to chromosome 6 in one reported case (Mori *et al.*, 1998), NFT parkinsonism as described by Rajput *et al.* (1989) and Alzheimer's disease-associated parkinsonism (Daniel and Lees, 1991). Detailed molecular studies of these conditions are not available, and it is not clear how closely they resemble the atypical PSP cases described in this series. However, although the neuropathology of parkin mutation cases may include NFT deposition, the atypical PSP cases in this series present at an older age (Lucking *et al.*, 2000). NFT parkinsonism as described by Rajput *et al.* (1989) predominantly involves the substantia nigra and locus coeruleus, but not other subcortical sites such as the subthalamic nucleus, which are characteristically involved in PSP. The atypical PSP cases described in this series had a more widespread subcortical NFT deposition. Unlike the atypical PSP cases, the Alzheimer's disease-associated parkinsonism cases were associated with sufficient amyloid plaque formation to be classified as Alzheimer's disease (Daniel and Lees, 1991).

The initial description of the tau genotype–PSP association was based on a pathologically defined series and indicated a 95% A0/A0 frequency among PSP patients. Subsequent series that have included clinically diagnosed cases have indicated a lesser association between A0/A0 and PSP, with the homozygote genotype frequency at ~70–80%. Our data indicate that when the strictest criteria for PSP diagnosis are used, and this includes pathological, biochemical and clinical information, the associated H1/H1 genotype frequency is 100%.

Although a relatively small number of cases have been studied in this series, the fact that there is an association between the *tau* H1H1 genotype and the deposition of normal PSP-type tau, regardless of the clinical presentation, may give some further indication of the role of the H1 haplotype in the pathogenesis of PSP. In FTDP-17 families PSP-type tau protein deposition occurs either in families with a coding mutation of exon 10 or in families with a splice-site mutation, which increases the splicing in of exon 10 at the RNA level (Hutton *et al.*, 1998). By extension, the pathogenesis of PSP may involve similar mechanisms, and some data support a change in the splicing of exon 10 in RNA analysis from post-mortem PSP tissue (Chambers *et al.*, 1999). This may be related in some way to the H1 haplotype.

The molecular pathological data in this series argue against a region-specific tau deposition response, as has been suggested may occur in Pick's disease (Delacourte *et al.*, 1998). In this study, tissue from the same area may contain either Alzheimer's disease-type tau or PSP-type tau, and in cases where both the brainstem and basal ganglia have been examined this has been found to be consistent. This argues that the type of tau deposited is disease- rather than region-specific. However, since the protein analysis is performed on homogenized brain areas we cannot exclude the possibility of involvement of different cellular populations within these areas, and this can only be properly examined by techniques such as *in situ* mRNA hybridization.

In summary, our data indicate that several discrete clinicopathological entities may lie within the spectrum of pathologically diagnosed PSP. However, the presence of the doublet PSP-tau deposition is correlated with a typical clinical presentation and the presence of the PSP susceptibility genotype. This study reinforces NFT diseases as an occasional pathological basis for a clinical Parkinson's disease-type syndrome and suggests possible pathways by which the susceptibility genotype may increase the likelihood of PSP development.

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Pathological tau burden and distribution distinguishes progressive supranuclear palsy-parkinsonism from Richardson's syndrome

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Clinical syndromes associated with progressive supranuclear palsy-tau pathology now include progressive supranuclear palsy-parkinsonism (PSP-P), in addition to classic Richardson's syndrome (RS) and pure akinesia with gait freezing (PAGF). Although pathological heterogeneity of progressive supranuclear palsy (PSP) has also been established, attempts to correlate this with clinical findings have only rarely provided conclusive results. The aim of this study was to investigate whether regional variations in the types of tau lesions or differences in overall tau load may explain the clinical differences between the RS, PSP-P and PAGF. Quantitative tau pathology assessment was performed in 17 brain regions in 42 cases of pathologically diagnosed PSP (22 RS, 14 PSP-P and 6 PAGF). Neurofibrillary tangles, tufted astrocytes, coiled bodies and thread pathology were quantitated and a grading system was developed separately for each region. Using these grades the overall tau load was calculated in each case. To establish a simplified system for grading the severity of tau pathology, all data were explored to identify the minimum number of regions that satisfactorily summarized the overall tau severity. The subthalamic nucleus, substantia nigra and globus pallidus were consistently the regions most severely affected by tau pathology. The mean severity in all regions of the RS group was higher than in PSP-P and PAGF, and the overall tau load was significantly higher in RS than in PSP-P ($P = 0.002$). Using only the grade of coiled body + thread lesions in the substantia nigra, caudate and dentate nucleus, a reliable and repeatable 12-tiered grading system was established (PSP-tau score: 0, mild tau pathology, restricted distribution; >7, severe, widespread tau pathology). PSP-tau score was negatively correlated with disease duration (Spearman's rho −0.36, $P = 0.028$) and time from disease onset to first fall (Spearman's rho −0.49, $P = 0.003$). The PSP-tau score in PSP-P (median 3, range 0–5) was significantly lower than in RS (median 5, range 2–10, Mann–Whitney U, $P < 0.001$). The two cases carrying the tau-H2 protective allele had the two lowest PSP-tau scores. We have identified significant pathological differences between the major clinical syndromes associated with PSP-tau pathology and the restricted, mild tau pathology in PSP-P supports its clinical distinction from RS. The grading system we have developed provides an easy-to-use and sensitive tool for the morphological assessment of PSP-tau pathology and allows for consideration of the clinical diversity that is known to occur in PSP.

Keywords: progressive supranuclear palsy; PSP; Richardson's syndrome; PSP-parkinsonism; tau

Abbreviations: CB = coiled body; NFT = neurofibrillary tangle; PAGF = pure akinesia with gait freezing; PSP = progressive supranuclear palsy; PSP-P = progressive supranuclear palsy-parkinsonism; RS = Richardson's syndrome; TA = tufted astrocyte

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Introduction

Progressive supranuclear palsy (PSP) is a degenerative disorder of the central nervous system with protean clinical manifestations. The classic clinical picture, originally described by Richardson is the most common clinical form (Richardson's syndrome or RS), with an insidious onset and relentlessly progressive postural instability and falls, gait disturbance, supranuclear vertical gaze abnormalities, pseudobulbar palsy, rigidity in extension and a dysexecutive syndrome (Richardson *et al.*, 1963; Steele *et al.*, 1964; Litvan *et al.*, 1996*a*; Williams *et al.*, 2005). We have recently defined a further clinical phenotype, PSP-parkinsonism (PSP-P), in which disease duration is longer and parkinsonism dominates the early clinical picture (Williams *et al.*, 2005). In contrast to RS these patients may show a moderate initial therapeutic response to levodopa, falls are delayed and if gaze palsy or dementia occur at all, they develop late in the course of the disease. PSP-tau pathology may also present as pure akinesia with gait freezing (PAGF), corticobasal syndrome or, exceptionally, with an isolated dementia (Imai *et al.*, 1993; Williams *et al.*, 2005; Tsuboi *et al.*, 2005; Josephs *et al.*, 2006).

The post-mortem diagnosis of PSP is dependent on the identification of neurofibrillary tangles (NFTs) and neuropil threads in basal ganglia and hindbrain structures (Litvan *et al.*, 1996*b*); tufted astrocytes (TAs) and coiled bodies (CBs) are other highly characteristic findings (Dickson, 1999). PSP is a primary tauopathy, in which tau dysfunction is regarded as central to the pathogenesis (Goedert, 2005). The predominance of 4-repeat tau isoforms in the neuronal and glial tau inclusions is also characteristic, although this is not included in the current operational diagnostic criteria (Komori *et al.*, 1998; Hauw, 2003; de Silva *et al.*, 2003). Pathological heterogeneity of PSP has also been reported (Braak *et al.*, 1992; Hof *et al.*, 1992; Mizusawa *et al.*, 1993; Verny *et al.*, 1996; Halliday *et al.*, 2000; Piao *et al.*, 2002; Williams *et al.*, 2005; Tsuboi *et al.*, 2005; Josephs *et al.*, 2005), but attempts to correlate this with clinical findings in PSP have only rarely revealed definitive correlations (Daniel *et al.*, 1995; Litvan *et al.*,1996*b*; Tsuboi *et al.*, 2005). Unlike Parkinson's disease and Alzheimer's disease, cases of 'incidental' PSP-tau pathology are unusual and pathological examples of 'early' PSP are rare, hampering any attempts to develop a pathological staging system. It is unknown whether the pathology in PSP follows a consistent topographical progression or whether a disease 'footprint' of regional susceptibility is established early with subsequent uniform progression of pathology in all affected structures. However, serial imaging studies confirm that the brainstem, in particular the midbrain, and frontal lobes bear the brunt of the disease (Paviour *et al.*, 2006).

We have already demonstrated an increased contribution by 3-repeat tau isoforms to the total insoluble-tau fraction in PSP-P, raising the possibility that biological differences influencing tau pathology may exist between PSP-P and RS (Williams *et al.*, 2005). In this study, we carried out a quantitative survey of different brain regions looking for regional differences in the tau load or variations in the type of tau lesions between the clinical PSP phenotypes. We have also applied a newly developed PSP-tau staging system in order to gain insight into the dynamics of the development of PSP-tau pathology.

Material and methods

Patients

We selected 42 cases from 102 pathologically diagnosed as PSP that were archived at the Sara Koe PSP Research Centre in the Queen Square Brain Bank for Neurological Disorders between 1992 and 2002. A systematic case note review was performed and the clinical features were recorded in a standardized fashion and cases were divided into three groups based on previously defined clinical criteria to allow for categorical analysis (Williams *et al.*, 2005). When falls, cognitive dysfunction, supranuclear gaze palsy, abnormalities of saccadic eye movements and postural instability were the predominant clinical features in the first 2 years of illness, RS was retrospectively diagnosed, whereas those without these features and with features including asymmetric bradykinesia of the limbs, a positive initial levodopa response, tremor or limb dystonia, were labelled as PSP-P. PAGF was defined when there was a history of gradual onset of freezing of gait or speech, absent limb rigidity and tremor, no sustained response to levodopa and no dementia or ophthalmoplegia in the first 5 years of disease. We aimed to select approximately equal numbers of cases classified as RS and PSP-P with additional representation of PAGF cases. In the most recent 21 brains, comprising 13 cases of RS, 7 of PSP-P and 1 of PAGF tissue blocks had been selected following a standardized protocol and these were therefore analysed first. A further 21 cases in which as many as possible of the appropriate anatomical areas were available for examination were randomly selected without reference to neuropathological details from a pool of 81 cases separated into RS, PSP-P and PAGF. This second group comprised nine cases of RS, seven cases of PSP-P and five cases of PAGF. In 26 cases tau haplotype data were available, and in 13 there were data regarding tau isoform profile in the pontine base (Williams *et al.*, 2005; Pittman *et al.*, 2005). The protocols for the retention and access to human tissue and clinical records at the Queen Square Brain Bank have approval from the London Multi-Centre Research Ethics Committee.

Pathological methods

Diagnostic procedures

Consent for brain donation was obtained from the patients prior to death and consent for post-mortem examination was obtained from the next of kin after death. The diagnosis of PSP was made using standard methods including immunohistochemical analysis using the AT8 anti-tau antibody (tau phospho-epitope Ser202/Thr205). The preliminary PSP pathological diagnostic criteria were applied which insist on the presence of NFTs, neuropil threads and glial tau pathology (Litvan *et al.*, 1996*b*).

For this study further neuropathological evaluation with a standardized approach was carried out to document Alzheimer pathology using Bielschowsky's silver impregnation and CERAD (Consortium to Establish a Registry for Alzheimer's Disease) criteria (Mirra *et al*., 1991). Aβ immunohistochemistry was also performed using an anti-Aβ antibody to document cerebral amyloid angiopathy (Revesz *et al*., 2003). Tau immunohistochemistry with the AT8 antibody was also used in the hippocampal formation to identify the presence of tau-positive grains in the hippocampal formation (Togo *et al*., 2002). The frontal lobe, lentiform nucleus and pons were examined for vascular pathology including small vessel atherosclerosis, lipohyalinosis, microaneurysm and arteriolosclerosis and their sequelae, such as lacunes, perivascular rarefaction and diffuse white matter attenuation of Binswanger's disease-type. Vascular pathology was graded as mild (occasional vessels affected), moderate (a significant proportion of the small vessels affected with few or no sequelae noted) or severe (a significant proportion of the small vessels affected with obvious sequelae). The presence of associated Lewy body pathology in the substantia nigra was assessed using α-synuclein immunohistochemistry.

Immunohistochemistry

Seven micrometres thick tissue sections were cut from tissue blocks of the anterior and posterior frontal cortex, parietal and temporal cortices, midbrain, pons and cerebellum for immunohistochemical analysis. Immunohistochemical staining for tau, α-synuclein and Aβ peptide (AT8, AutogenBioclear, 1 : 600; α-synuclein, Novocastra, 1 : 50; Aβ peptide, Dako, 1 : 100) was performed using a standard avidin–biotin method.

Regional pathological examination and quantification

In each case quantitative assessment of the tau pathology was carried out by one rater (DRW), blinded to the clinical features, in 17 brain regions that have previously been documented to show variability in PSP or were predicted to contribute to the clinical features of disease. The cortex and white matter in anterior and posterior frontal lobes, parietal lobe and temporal lobe, internal and external globus pallidus, putamen, caudate nucleus, subthalamic nucleus, substantia nigra, pontine nuclei, dentate nucleus and cerebellar white matter were used. In each region AT8 positive NFTs, TAs and oligodendroglial CBs were counted separately in seven randomly placed microscopic fields using a ×20 objective. Thread-like positivity was counted within a 10 × 10 graticule in the same field (×20 objective) and added to the CB score to give a CB plus thread (CB+Th) score. This enabled the classification of all immunoreactive lesions that were not obviously part of NFTs or TAs. Absolute counts were converted to a five-point grading scale that was developed for each region after the distribution of lesion counts was examined. Counted values were plotted along the *x*-axis and ranges for grades 0 to 4 were assigned on the *y*-axis. Grade 0 was reserved for when tau-positive pathology was absent. If the distribution of counts fitted a logarithmic curve the upper limit of grade 4 was set as the highest number counted, grade 1 the lowest count above zero and grades 2, 3 and 4 evenly spaced between these markers on a log scale along the *y*-axis. If the distribution of counts suggested that a linear model was more appropriate the upper limit of grade 4 was set at the highest value counted and grades 1–3 were set at 25, 50 and 75% of this highest value. In regions where the range included less than fou values, absolute counts were used.

The repeatability of all counts was assessed by the same rate (DRW) by re-counting four (10%) cases. The intra-rater standar deviation (IRSD) was calculated as follows: IRSD = $SD(R_{m1} - R_{m2})/\sqrt{2}$, where SD is standard deviation, R_{m1} i median grade at first reading and R_{m2} is median grade a second reading.

Regions representative of overall tau severity

All graded data were explored to identify the minimum number o regions that satisfactorily summarized the overall tau severity Grades from the temporal lobe grey matter were excluded t remove the influence of Alzheimer-type pathology on the analysis First, the overall tau severity was determined in each case, b adding the grades of all lesions in all regions together. Next, th median grades for NFT, TAs, CBs and CB+Th were calculated fo each region in each case (see example, Fig. 1). After choosin regions with the greatest range of grades, different combination of regions and lesion types were tested for correlation with overal tau severity. It was found that the sum of grades for CB+T pathology in the substantia nigra, caudate and dentate nucleu best correlated with the overall tau severity (see section 'Results') This number designated the PSP-tau score (substantia nigr CB+Th grade + caudate CB+Th grade + dentate CB+Th grade had a range from 0 to 12. The Friedman test was used to asses whether increments in the PSP-tau scores were associated wit statistically significant increases in overall tau severity.

Neuropathological grading of tau severity

So that the repeatability of the pathological data used to produc the PSP-tau score could be tested, images representative of grade 1 to 4 of CB+Th pathology for the three regions of interest wer

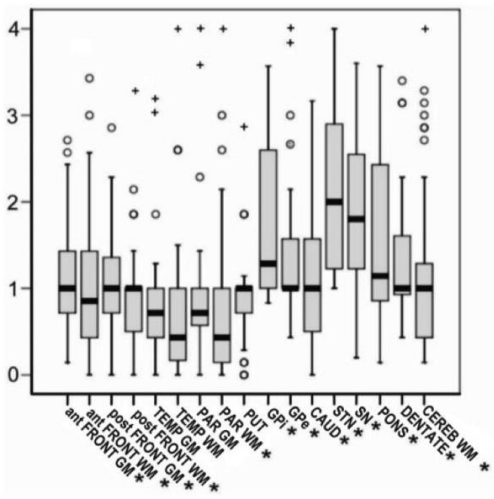

Fig. 1 Coiled body + thread pathology grades in different brain regions; median, interquartile ranges; ° outliers; + extreme outliers; * regions used to test for correlation of overall tau.

obtained and used to produce a visual aid to grading (Fig. 2). This aid only, was used independently by two neuropathologists (TR and JH) as a guide to grading CB+Th pathology in the substantia nigra, caudate and dentate nucleus. While blinded from the clinical details, they were asked to grade the CB+Th pathology in the 34 cases of PSP with a complete data set (RS 21, PSP-P 12 and PAGF 1). The neuropathologists were asked to examine five to seven microscopic fields (×20 objective) in the dentate nucleus, caudate and substantia nigra. They were instructed to consider all sub-regions of the substantia nigra represented in the section and informed that all tau-positive lesions except NFTs and TAs were to be evaluated to give the CB+Th grade. Grade 0 was given when there were no pathological lesions, and was not included on the visual aid. Finally PSP-tau scores were calculated using the sum of median scores from all the three sampled regions (substantia nigra CB+Th grade + caudate CB+Th grade + dentate nucleus CB+Th grade), giving a score with a minimum of 0 and maximum of 12. Agreement between these two raters was assessed by calculating the weighted kappa and applying Landis and Koch's categorization of responses (Landis and Koch, 1977).

Distribution of pathological tau

The regional distribution of PSP-tau pathology was compared between cases of different severity. Cases were separated according to their PSP-tau score (0–1, 2–3, 4–5, 6–7 and >7) and median values of CB+Th were calculated for each brain region in each group.

Statistical methods

Spearman's correlation calculation was used to determine the relationship between the PSP-tau score and disease duration, age of onset, age at death, time from disease onset to first fall and time from disease onset to supranuclear gaze palsy. The correlation between PSP-tau score and the severity of tau lesions in each region (grade and absolute counts) was also examined using Spearman's calculation. The significance level for this test was set at 0.01 because of the multiple comparisons. For other data univariable analyses using χ^2 for categorical and two-tailed t test or the Mann–Whitney U test, as appropriate, for continuous variables were applied with a significance level of 0.05. All statistical analysis was performed using SPSS for Windows (version 12.0.1).

Results

Forty-two patients (26 men, 16 women) were included in the study. The mean age of onset was 66.5 years (range 44.4–87.5), the mean age at death was 75.6 years (60.9–95.8) and the mean disease duration was 9.1 years (1.2–17.3). Additional pathological findings are recorded in Table 1. There was no difference between the RS and PSP-P groups in the prevalence or severity of the CERAD plaque score, small vessel pathology, cerebral amyloid angiopathy or prevalence of tau-positive grains.

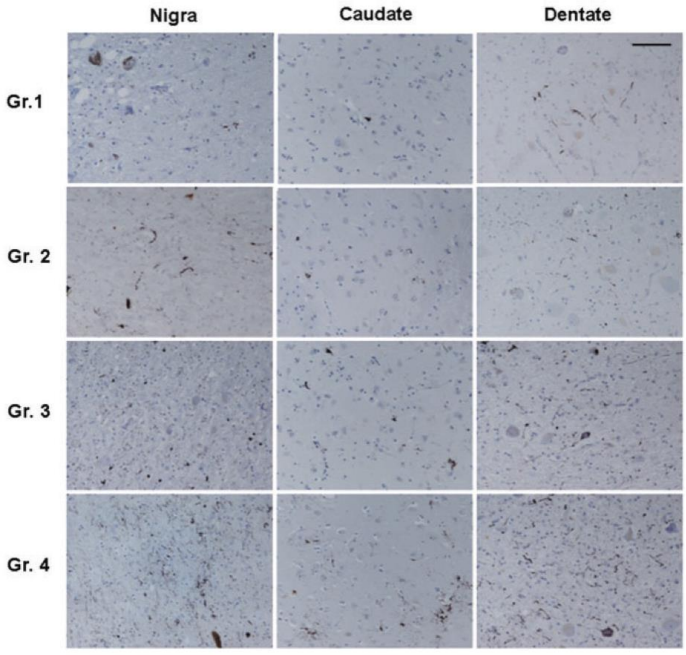

Fig. 2 Visual guide for scoring pathology severity in three regions of interest. Grade 0 was reserved for there was no AT8 positivity; bar represents 77 μm on all panels (×20 magnification).

PSP-tau pathology

The diagnosis of PSP was confirmed in all the cases by the presence of typical PSP-tau lesions including NFTs, TAs, CBs and neuropil threads in the expected distribution. The intra-rater standard deviation of the regional analysis was 0.012, implying that the variability of counting was <2% and was thus highly repeatable.

The number of pathological lesions per microscopic field varied substantially between regions and between cases, although in no case was the subthalamic nucleus, substantia nigra or internal globus pallidus spared. NFTs were most abundant in the basal ganglia and brainstem structures and TAs in the cortices, putamen and caudate nucleus. CB+Th were the major contributor to the overall tau load and were most numerous in the subthalamic nucleus (median 283/microscopic field, range 8–1710), substantia nigra (median 186/microscopic field, range 0–1430) and internal globus pallidus (median 49/microscopic field, range 0–720), and least dense in the cortical regions.

Table 1 Additional pathological findings by clinical group

Number	RS 23	PSP-P 13	PAGF 6
CERAD neuritic plaque score			
Negative	10 (45)	6 (46)	2 (33)
Positive	13 (55)	7 (54)	4 (67)
Sparse	5 (21)	3 (23)	2 (33)
Moderate	7 (30)	3 (23)	2 (33)
Frequent	1 (4)	1 (8)	0
Tau-positive grains	4 (18)	6 (46)	0
Lewy body pathology	1 (4)	0	0
Small vessel pathology	7 (30)	6 (46)	0
Mild	6 (26)	5 (38)	0
Moderate	0	1 (8)	0
Severe	1 (4)	0	0
Cerebral amyloid angiopathy	3 (13)	3 (23)	1 (17)

Note: Percentages are shown in brackets.

In the cases with the lowest density of lesions, tau pathology was limited to the subthalamic nucleus, substantia nigra and internal globus pallidus, with sparse tau immunoreactivity in the posterior frontal cortex and white matter, and few NFTs in these structures. As the severity of CB+Th pathology in the subthalamic nucleus, substantia nigra and internal globus pallidus increased, there was a concurrent increase in the density and severity of tau immunoreactivity in the pontine nuclei, dentate nucleus, cerebellar white matter and frontal cortex. The parietal lobe was not affected in cases with milder tau pathology, but in those with severe CB+Th pathology in the basal ganglia it was severely affected. Alzheimer changes lay within the expected range for the age groups, but Lewy body pathology was less frequent than previously reported (Braak *et al.*, 1992; Tsuboi *et al.*, 2001).

The mean regional tau-severity was higher in RS than PSP-P and PAGF in all brain regions. The difference with PSP-P was significant in all regions except the putamen (Mann–Whitney U test, $P=0.15$) and subthalamic nucleus (Mann–Whitney U test, $P=0.77$) (Fig. 3). Tau pathology was significantly more severe in RS than PAGF in the parietal cortex (Mann–Whitney U test, $P=0.022$), pontine nuclei (Mann–Whitney U test, $P=0.003$), dentate nucleus (Mann–Whitney U test, $P=0.01$) and cerebellar white matter (Mann–Whitney U test, $P=0.023$). Total tau load ($\sum$ of grades for all lesions in all regions) was higher in the RS group (median 155) than in the PSP-P group (median 116, Mann–Whitney U test, $P=0.002$) (Fig. 4). The PAGF group was not analysed further because of the small number of cases with a complete data set.

Grading of PSP-tau severity

The PSP-tau score ($\sum$ CB+Th grade in the substantia nigra, caudate and dentate nucleus) correlated significantly

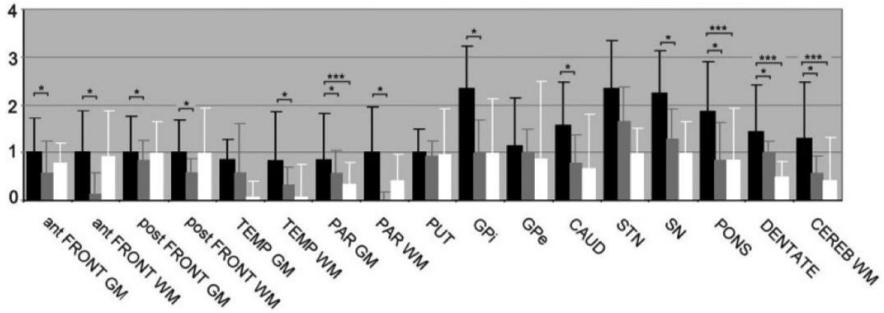

Fig. 3 Median regional tau severity in RS (black), PSP-P (grey) and PAGF (white). 1 standard deviation indicated by error bars. *RS versus PSP-P, Mann–Whitney U, $P<0.05$; ***RS versus PAGF, Mann–Whitney U, $P<0.05$. ant, anterior; post, posterior; GM, grey matter; WM, white matter; FRONT, frontal lobe; TEMP, temporal lobe; PAR, parietal lobe; PUT, putamen; GPi, internal globus pallidus; GPe, external globus pallidus; CAUD, caudate; STN, subthalamic nucleus; SN, substantia nigra; PONS, pontine nuclei; DENTATE, dentate nucleus; CEREB, cerebellar.

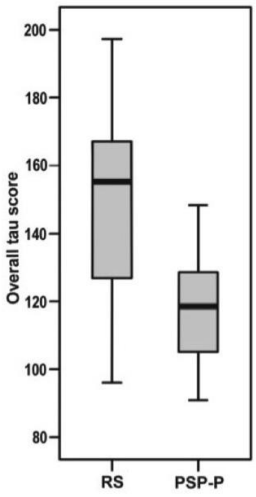

Fig. 4 Overall tau severity (sum of grades for all tau lesions in all regions) according to clinical group. Median (Mann–Whitney U test, $P = 0.002$) and interquartile ranges.

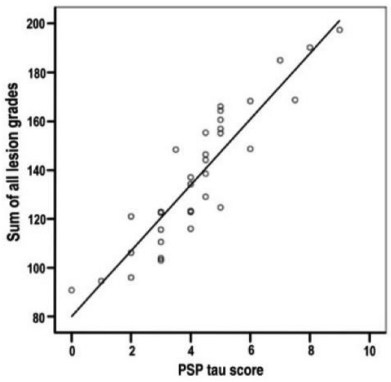

Fig. 5 Correlation between PSP-tau score and sum of all tau grades (Spearman's rho 0.93, $P < 0.001$).

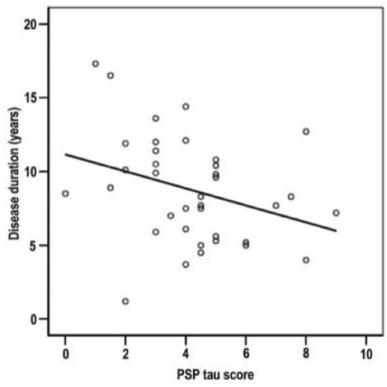

Fig. 6 PSP-tau score versus disease duration (Spearman's rho −0.36, $P = 0.028$).

with the overall CB+Th severity ($\sum$ CB+Th grades in all 17 regions, Spearman's rho 0.89, $P < 0.001$) and also highly correlated with the overall lesion severity comprising all lesion types ($\sum$ all lesion grades in all 17 regions, Fig. 5, Spearman's rho 0.93, $P < 0.001$) in the 34 cases with a complete data set. There was a significant positive correlation (Spearman's rho, $P < 0.01$) between the PSP-tau score and lesion grades in 70% of regions and lesion counts in 63% of regions (supplementary material). PSP-tau score correlated with NFT grades in 36% of brain regions, CB grades in 100% and CB+Th grades in 94% of regions (all, Spearman's rho $P < 0.01$). No other combination of regional grades correlated with more regions. Each increment in the PSP-tau score (tau score 0 to 10—this latter being the highest observed) was significantly different from the previous according to the mean ordinal rank of tau severity (Friedman's χ^2, 23.7, $P = 0.005$). The weighted kappa measuring agreement between two independent pathologists using the visual aid and instructions for grading was 0.71, which is 'very' good inter-rater agreement (Landis and Koch, 1977).

The PSP-tau score did not correlate with age at symptom onset or age at death, but there was a negative correlation between PSP-tau score and disease duration (Spearman's rho −0.36, $P = 0.028$) (Fig. 6). Time from disease onset to first fall (Spearman's rho −0.49, $P = 0.003$) was negatively correlated with PSP-tau score. There was a modest, but significant correlation between tau isoform ratio (4-repeat tau:3-repeat tau) in the few cases with this data (Spearman's rho 0.56, $P = 0.048$). The only two cases that did not have the H1/H1 PSP susceptibility genotype (H2/H2 and H2/H1) had the lowest PSP-tau scores (0 and 1, respectively). The three cases homozygous for the H1c haplotype did not have a significantly higher tau score than cases with other genotypes. No cases classified as PSP-P had a PSP-tau score of more than 5 (median 3), and there was a significant difference between the median PSP-tau scores for RS (median 5, versus PSP-P, Mann–Whitney U test, $P < 0.001$) (Fig. 7).

Distribution of PSP-tau pathology

As our findings suggested that the PSP-tau score (sum of CB+Th grades in substantia nigra, caudate and dentate nucleus) was a reasonable surrogate marker for pathological

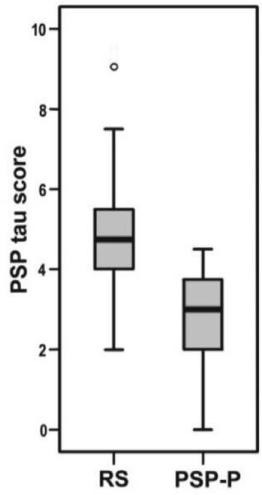

Fig. 7 PSP-tau score according to clinical group, median (Mann–Whitney U test, $P < 0.001$) and interquartile ranges.

disease severity with significant clinical correlations, the relationship between this surrogate marker for pathological severity and the distribution of lesions throughout all 17 brain regions was further examined. Cases were grouped according to PSP-tau score (PSP-tau score 0–1, 2 cases; 2–3, 9 cases; 4–5, 16 cases; 6–7, 3 cases; >7, 3 cases) and median values of CB+Th for each region were calculated (Fig. 8). Pathological features noted, in addition to the graphically displayed median data for CB+Th, are discussed according to PSP-tau score.

Scores 0–1: involvement limited to pallido-luyso-nigral distribution with sparse involvement of the pre-motor cortex: Regions caudal to the substantia nigra had little or no tau pathology, including the dentate nucleus, cerebellar white matter and the pontine nuclei. In these cases the parietal cortex was spared from tau lesions and the anterior frontal lobe had very few lesions.

Scores 2–3: moderate involvement of basal ganglia, pontine nuclei and dentate nucleus in the absence of parietal lobe lesions: The severity of the previously described lesions was increased and in particular the number of tau positive lesions in the subthalamic nucleus and globus pallidus was increased. Caudal regions, including the dentate nucleus and pontine nuclei, were affected and in some cases the cerebellar white matter was mildly involved. There were NFTs, TAs and thread pathology in the posterior frontal lobe, but the anterior frontal lobe was rarely involved.

Scores 4–5: more severe involvement of the basal ganglia and dentate nucleus with involvement of the frontal and parietal lobes: Brains with scores of 4 or 5 were most numerous in this study. The internal globus pallidus, subthalamic nucleus, substantia nigra, pontine nuclei, dentate nucleus and cerebellar white matter were more severely affected, but there was no increase in CB+Th pathologies in the external globus pallidus. Cortical regions, including the frontal and parietal lobes, were consistently moderately affected.

Scores 6–7: moderately severe pathology in the basal ganglia, pontine nuclei, parietal and frontal lobes: In the internal globus pallidus numerous NFTs, threads and CBs were present, and the severity of all lesions, except NFTs, was equally severe in the external globus pallidus. The caudate and putamen remained only moderately affected, similar to the lower scored brains, but the involvement of caudal structures was more severe, in particular the cerebellar white matter. In these brains the neocortical regions, except the temporal lobe, had moderately severe NFTs, TAs, thread pathology and CBs.

Score >7: severe involvement of the subthalamic nucleus, substantia nigra, internal globus pallidus as well as neocortical areas, pontine nuclei and cerebellar structures: In the three cases in this category there was severe pathology throughout the regions examined, with the exception of the caudate, putamen and temporal lobe where there was very little increase in the numbers of tau lesions as the score increased above 2.

Discussion

The higher contribution by 3-repeat tau isoforms to the insoluble tau fraction in PSP-P raised the possibility that the biological differences determining different clinical PSP sub-types might also affect the severity and distribution of the histopathological lesions (Williams *et al.*, 2005). We have indeed demonstrated pathological differences between the two major clinical sub-types, with the RS group having a significantly higher total PSP-tau burden than the PSP-P group. The quantitative data for the overall severity of PSP-tau pathology can be assessed reliably by using a 12-tiered scoring system that takes into account the tau burden in oligodendroglia and threads in three anatomical regions: the substantia nigra, caudate and dentate nucleus. This can also be reproduced consistently when only a visual aid, akin to those used for the CERAD diagnosis of Alzheimer's disease (Mirra *et al.*, 1991) and dementia with Lewy bodies (McKeith *et al.*, 2005), is used for grading. Having assigned a PSP-tau score to each case, an excellent correlation was found with the overall tau-load determined by the morphometric data. Further analysis also revealed important associations between PSP-tau scores and clinical, biochemical and genetic features of the cases. Those with RS had a significantly higher PSP-tau score than the PSP-P cases, and no PSP-P case had a score of more than 5. In addition, we established that with increments in PSP-tau scores tau deposition also becomes more widespread indicating that PSP-P is not only associated

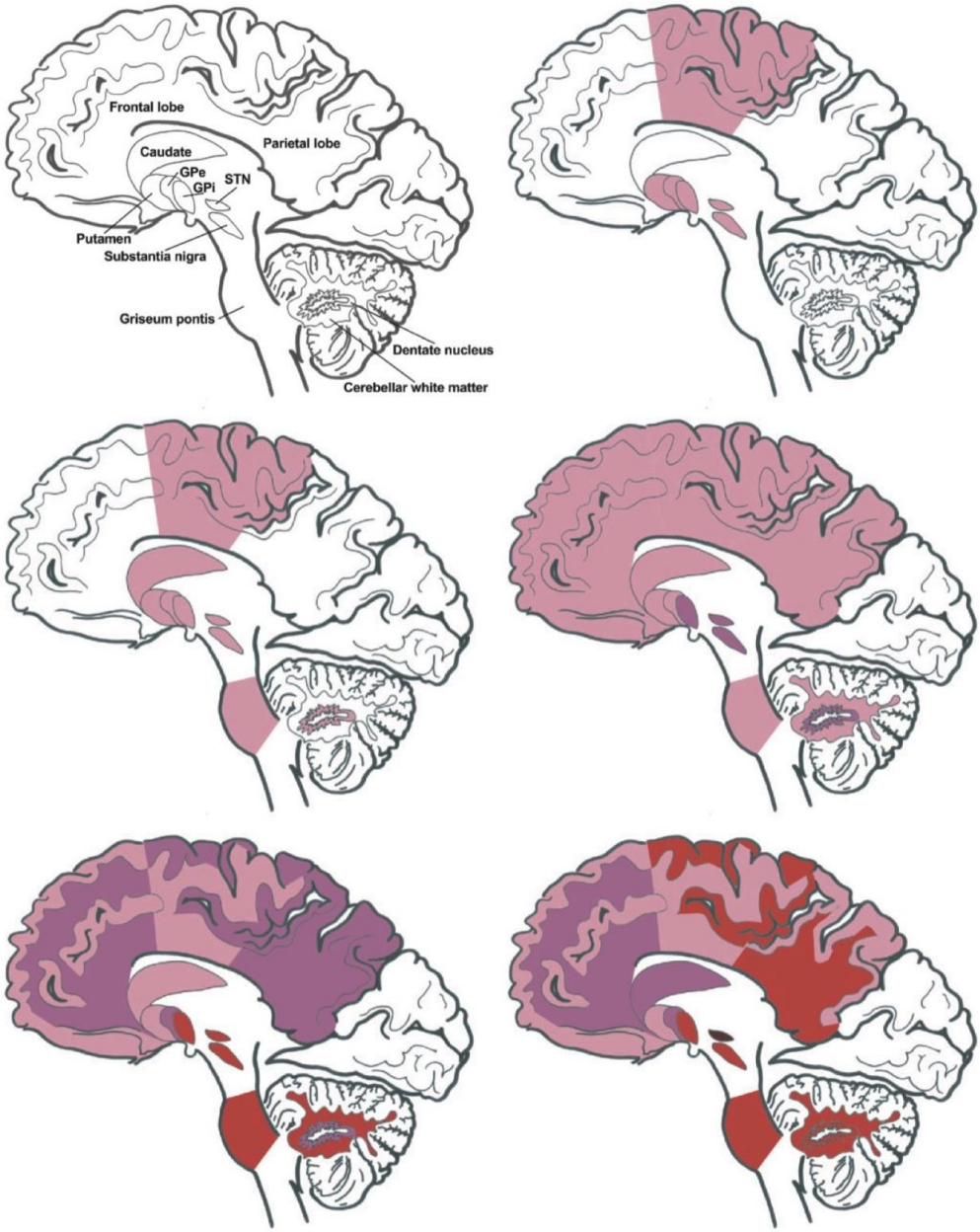

Fig. 8 Distribution of median coiled body + thread tau pathology, according to PSP-tau score. Colour/median grade per PSP-tau score: pink/grade 1; purple/grade 2; red/grade 3; brown/grade 4. A = legend; B = PSP-tau scores 0–1; C = PSP-tau scores 2–3; D = PSP-tau scores 4–5; E = PSP-tau scores 6–7; F = PSP-tau scores >7.

with a lower tau burden, but also that this takes place in a topographically more restricted pattern. Disease duration was shortest in patients with the most severe tau pathology, and therefore the highest PSP-tau score and topographically most extensive tau pathology. Tau severity, according to this score, correlated with 4-repeat tau:3-repeat tau ratio. Patients who carried the H2 PSP-protective allele had the lowest tau scores.

Although differences in the severity of pathology in some brain regions have previously been correlated with particular clinical features, heterogeneity in regional tau distribution has made qualitative and quantitative comparisons between pathology and clinical features problematic (Daniel *et al.*, 1995; Verny *et al.*, 1996; Revesz *et al.*, 1996; Bigio *et al.*, 1999; Halliday *et al.*, 2000; Tsuboi *et al.*, 2005; Josephs *et al.*, 2006). We used an unbiased, region-specific and lesion-specific grading system to overcome these difficulties and to allow for regional comparisons to be made. The severity of CB+Th pathologies were the most useful in separating cases of PSP and the PSP-tau score correlated well with the majority of other pathological lesions, but not NFTs in the striatum, substantia nigra or subthalamic nucleus (see supplementary material). Unlike NFTs, there is no convincing evidence that threads, representing accumulation of PSP-tau in both neuronal and oligodendroglial processes (Probst *et al.*, 1988; Ikeda *et al.*, 1994; Dickson, 1999), decrease in density in PSP as the disease progresses (Halliday *et al.*, 2000), making their post-mortem analysis more likely to reflect dynamic changes during the course of the disease.

We have previously shown that amongst the clinical features of patients with PSP-tau pathology, a number of distinct clinical syndromes can be identified (Williams *et al.*, 2005). The close association between the severity of the PSP-tau scores, as a measure of the PSP-tau load, and the clinical sub-types also suggests that they are a sensitive surrogate marker of the functional and structural changes that accompany the underlying neurodegenerative processes and determine the different PSP phenotypes. Others have also found that the severity of tau accumulation, and neuronal loss, in the substantia nigra pars reticulata (Halliday *et al.*, 2000), mesencephalon (Juncos *et al.*, 1991) and the pontine nucleus raphe interpositus (Revesz *et al.*, 1996), is higher in patients with gaze palsy (RS) compared to those without (mostly PSP-P). However, the current findings suggest these localized changes mirror neurodegeneration in a number of other susceptible regions, and are part of more severe and widespread disease in the brain. This notion is supported by several reports of an association between the severity of tau changes in cortical regions and cognitive dysfunction in PSP and by our finding of more severe cortical pathology in RS (Bergeron *et al.*, 1997; Tsuboi *et al.*, 2005; Josephs *et al.*, 2006). In contrast, patients who present with parkinsonism without gaze palsy, early falls and dementia have more moderate pathological tau accumulation, in a more restricted pattern of distribution.

The general pattern of distribution of tau pathology in PSP appears remarkably consistent despite the broad spectrum of clinical features recorded during life. No case of 'incidental' PSP-tau has been included in this analysis, and, to our knowledge, definitive reports of such cases have not been made. In their absence, and without a substantial number of cases with early PSP, attempts to determine the dynamics of topographical evolution of tau pathology cannot be considered. However, we have constructed a model that may represent the disease 'footprint' of relative susceptibility to regional pathological tau accumulation in PSP. This model identifies the subthalamic nucleus, substantia nigra and globus pallidus as the most susceptible regions and that other regions lag behind in severity in a gradient from the posterior frontal lobe, dentate nucleus, cerebellar white matter, pontine nuclei, caudate, to the anterior frontal and parietal lobes. The significant negative correlation between the PSP-tau score and disease duration suggests that more fulminant disease affects more regions, more severely from disease onset and contributes to an earlier death. The factors that influence the severity and extent of PSP-tau pathology are unknown, but the genetic background and, in particular, possession of the H1c tau sub-haplotype, may be important (Baker *et al.*, 1999; Pittman *et al.*, 2005). Our observation that the patients with at least one H2 allele accounted for the two mildest cases of tau accumulation is of interest given recent suggestions that the H2 tau haplotype not only lacks an association with PSP, but also that it contributes to protection from the disease (Pittman *et al.*, 2005). The least severe pathology also tended to have the lowest ratio of 4-repeat tau:3-repeat tau in the pontine base. Recent findings suggest that the presence of the *MAPT* H1c sub-haplotype favours the increased production of 4-repeat tau in controls and possibly its accumulation in disease (Myers *et al.*, 2006). The small number of cases homozygous for H1c tau sub-haplotype in our study did not have a significantly higher severity of tau pathology implying that other modifying factors may influence the effect of genetic susceptibility.

Our data are also consistent with the notion that regions predicted to be most susceptible to PSP-tau neurodegeneration (subthalamic nucleus, substantia nigra and globus pallidus) have the lowest threshold for disease manifestation and in the context of protective factors, such as the H2 allele for example, may remain the only severely affected regions. Other related tauopathies, such as post-encephalitic parkinsonism and parkinsonism dementia complex of Guam have overlapping pathological features (Geddes *et al.*, 1993), and recent data suggest that genetic factors that contribute to the Guam neurodegenerative disease risk may also, at least in part, be similar to those in PSP (Sundar *et al.*, 2007). The differences in the type, ultrastructural and biochemical characteristics of the tau

pathologies of these diseases and PSP that are also documented are, however, consistent with the presumed different aetiologies of these conditions (Geddes *et al.*, 1993; Buee-Scherrer *et al.*, 1995; Litvan *et al.*, 1996*b*; Mawal-Dewan *et al.*, 1996; Buee-Scherrer *et al.*, 1997; Oyanagi *et al.*, 2001; Oyanagi, 2005; Williams, 2006). Nevertheless the similarities in the distribution of the pathological lesions in post-encephalitic parkinsonism and parkinsonism dementia complex of Guam that overlap with the range described in the present work, raise the possibility of similar factors contributing to regional tau susceptibility in these diseases particularly in the substantia nigra, subthalamic nucleus and pallidum (Geddes *et al.*, 1993; Oyanagi *et al.*, 1997, 2000, 2001).

The grading system used in this study is a practical and sensitive tool for the morphological assessment of PSP-tau pathology and takes into account the clinical, biochemical and genetic diversity that is known to occur in PSP. Although the relationship between neuronal loss and PSP-tau pathology remains to be determined, the proposed model provides a framework for further evaluating the cellular factors that contribute to PSP-tau neurodegeneration, and the clinical and biochemical manifestations of the disease. A pathological distinction between PSP-P and RS can be made, which together with the biochemical differences gives weight to previous attempts of separating these two PSP phenotypes clinically. Future research identifying genetic and other disease markers may also help to underpin the notion that PSP-P is a distinct clinicopathological entity, albeit with a close relationship with RS. In the meantime the pathological distinction between PSP-P and RS emphasizes the need to identify these patients in the clinic and that research in PSP should also take into account the biological differences that exist between these clinical phenotypes.

Supplementary material

Supplementary material is available at *Brain* online.

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Characteristics of progressive supranuclear palsy presenting with corticobasal syndrome: a cortical variant

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Characteristics of progressive supranuclear palsy presenting with corticobasal syndrome: a cortical variant

Aims: Since the first description of the classical presentation of progressive supranuclear palsy (PSP) in 1963, now known as Richardson's syndrome (PSP-RS), several distinct clinical syndromes have been associated with PSP-tau pathology. Like other neurodegenerative disorders, the severity and distribution of phosphorylated tau pathology are closely associated with the clinical heterogeneity of PSP variants. PSP with corticobasal syndrome presentation (PSP-CBS) was reported to have more tau load in the mid-frontal and inferior-parietal cortices than in PSP-RS. However, it is uncertain if differences exist in the distribution of tau pathology in other brain regions or if the overall tau load is increased in the brains of PSP-CBS. **Methods:** We sought to compare the clinical and pathological features of PSP-CBS and PSP-RS including quantitative assessment of tau load in 15 cortical, basal ganglia and cerebellar regions. **Results:** In addition to the similar age of onset and disease duration, we demonstrated that the overall severity of tau pathology was the same between PSP-CBS and PSP-RS. We identified that there was a shift of tau burden towards the cortical regions away from the basal ganglia; supporting the notion that PSP-CBS is a 'cortical' PSP variant. PSP-CBS also had less severe neuronal loss in the dorsolateral and ventrolateral subregions of the substantia nigra and more severe microglial response in the corticospinal tract than in PSP-RS; however, neuronal loss in subthalamic nucleus was equally severe in both groups. **Conclusions:** A better understanding of the factors that influence the selective pathological vulnerability in different PSP variants will provide further insights into the neurodegenerative process underlying tauopathies.

Keywords: alien limb, corticobasal syndrome, progressive supranuclear palsy, Richardson's syndrome, tau

Introduction

The classical presentation of progressive supranuclear palsy (PSP), now known as Richardson's syndrome (PSP-RS), includes as cardinal features the early onset of postural instability with falls backwards, vertical supranuclear gaze palsy (VSGP) including downgaze and frontal subcortical cognitive impairment [1–3]. Other well-recognized clinical variants of PSP are PSP-parkinsonism

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(PSP-P) [2,4], pure akinesia and gait freezing (PSP-PAGF) [5,6], primary non-fluent aphasia (PSP-PNFA) [7–10], behavioural variant of frontotemporal dementia (PSP-bvFTD) [11] and corticobasal syndrome (PSP-CBS) [12]. Clinicopathological studies have since demonstrated a close correlation between topographical severity of tau pathology and clinical phenotypes of PSP. For instance, severe tau pathology was identified in the inferior frontal gyrus in PSP-PNFA [8] and frontal and temporal cortices in PSP-bvFTD [13]. In contrast, cortical tau was found to be very mild in the PSP-PAGF subtype [5,6]. Similar clinicopathological correlation was also identified in another closely related 4-repeat (4R) tauopathy, corticobasal degeneration (CBD) and its clinical phenotypes [14].

Corticobasal syndrome (CBS) describes progressive clumsiness and loss of function of one hand due to apraxia, an alien limb, cortical sensory loss, dystonia and levodopa-unresponsive rigidity, and it was initially described as the distinctive clinical presentation of CBD [15,16]. Since its original description, multifarious other pathologies have been linked to a CBS presentation [7,17,18]. From the archives of the Queen Square Brain Bank for Neurological Disorders (QSBB), we showed that the most common underlying pathology for CBS is PSP (6 of 21) rather than CBD (5 of 21); however, only 4% of all pathologically diagnosed PSP cases ($n = 227$) had a CBS presentation (PSP-CBS) [17]. Previously, Tsuboi *et al.* quantified tau load in four selected cortical regions including cingulate gyrus, mid-frontal cortex, motor cortex and inferior-parietal cortex in three PSP-CBS cases and eight randomly chosen PSP-RS cases [12]. They reported an increased tau pathology in the mid-frontal and inferior-parietal cortices in PSP-CBS compared with PSP-RS and concluded that the CBS presentation of PSP was either caused by a concurrent cortical pathology from a secondary process such as Alzheimer's disease or primary PSP tau pathology involving the cortical regions [12]. Nevertheless, it is uncertain if differences exist in the distribution of tau pathology in other brain regions or if the overall tau load is increased in the brains of PSP-CBS. It is noteworthy that imaging studies have identified predominant focal grey matter loss on voxel-based morphometry in premotor cortex, posterior superior frontal lobe and supplementary motor area and relatively preserved brain stem grey matter in cases with PSP-CBS [19]. We therefore hypothesized that the distribution of tau pathology in PSP-CBS may resemble the distribution of grey matter loss identified by *in vivo* imaging in voxel-based morphometry.

The aims of this study were: (i) to validate the findings reported by Tsuboi *et al.* in a significantly larger cohort of PSP-CBS cases and to quantitatively assess tau distribution in more cortical regions and other brain regions including the basal ganglia, brainstem and cerebellum; (ii) to determine the cellular lesions which contribute to the tau pathology were characteristic of PSP pathology rather than Alzheimer-type neurofibrillary tangle pathology; and (iii) to assess neuronal loss of the substantia nigra and subthalamic nuclei and pathological involvement of the corticospinal tract.

Materials and methods

Cases

Of the 227 PSP cases available in the QSBB archives between 1988 and 2010, nine had received a final clinical diagnosis of CBS/CBD by a neurologist during life (PSP-CBS, 3.9% of all PSP cases). An additional case, seen and diagnosed pathologically at the University of Nottingham, was also included. These 10 PSP-CBS cases were matched with 10 PSP-RS control cases for disease duration and age at death. The brain donor programme of the QSBB was approved by a London Multi-Centre Research Ethics Committee and tissue is stored for research under a license from the Human Tissue Authority.

Medical record review

Systematic retrospective review of the medical records was carried out by one of us (H. L.). All patients were assessed by at least one neurologist during life. Symptoms and clinical signs were recorded as being absent if they were not reported in the case notes. When the onset was not recorded, the onset was taken as the time when the particular clinical feature was first mentioned in the notes. If there were conflicting clinical features, the findings of the neurologist took precedence. The definitions for each selected clinical feature have been described previously [17].

Pathological material

The neuropathological diagnosis of PSP was confirmed in all 20 cases (T. R. and J. L. H.). Immediately after *post mortem* the brains were divided in the mid-sagittal plane. One half, chosen randomly, was sliced and tissue blocks were frozen and stored at −80°C, while the other half was

immersed and fixed in 10% neutral formalin for 3 weeks before neuropathological examination. Tissue blocks were taken using standard protocols. Established pathological diagnostic criteria for PSP were used, requiring the presence of neurofibrillary tangles (NFTs), neuropil threads (NTs) and glial tau pathology in different brain regions including the cerebral cortex, striatum, globus pallidus (GP), subthalamic nucleus (STN), midbrain, pons and cerebellum together with neuronal loss and gliosis in basal ganglia and brainstem and cerebellar nuclei [20–23]. In each case 8-μm-thick tissue sections cut from the paraffin blocks and stained with haematoxylin and eosin (H&E) were used to assess neuronal loss and gliosis in the basal ganglia and substantia nigra. Immunohistochemistry with antibodies to phosphorylated tau (AT8; BioScience Life Sciences; 1:600), 3-repeat (3R) tau and 4R tau (Upstate/Millipore; 3R tau: RD3; 1:2000; 4R tau: RD4; 1:200) [24], microglia (CD68; Dako; PG-M1; 1:75), αB-crystallin (Novocastra; G2JF; 1:300), amyloid-β (Aβ) peptide (Dako; 6F/3D; 1:100) and α-synuclein (Vector Laboratories; KM51; 1:50) was performed using a standard avidin-biotin method as previously described [25].

Additional pathologies were documented. Argyrophilic grain disease was identified by AT8 and αB-crystallin immunohistochemistry [26] while Aβ cortical plaque pathology was characterized using the modified CERAD (Consortium to Establish a Registry for Alzheimer's disease) criteria [27]. Alzheimer's type NFT pathology was determined using AT8 immunohistochemistry for Braak and Braak staging [28]. The presence of incidental Lewy body disease [29], cerebrovascular disease [30] and cerebral amyloid angiopathy (CAA) [31] was documented. Only cases with limited Alzheimer-type neurofibrillary tangle pathology of Braak and Braak stage III or less were recruited to avoid confounding the analysis of PSP-related tau pathology.

Regional tau quantification with image analysis

Using coded slides, quantitative assessment of tau pathology, comprising all tau-positive structures including NFTs, pretangles (PreTs) NTs, tufted astrocytes (TAs) and coiled bodies (CBs) was performed by one rater (H. L.). Fifteen brain regions, which are known to be affected in PSP and whose involvement is predicted to contribute to the clinical features, were selected; the posterior frontal cortex including the motor strip, cerebral cortex and subcortical white matter of the middle frontal gyrus (level: 1 cm behind the temporal pole), middle temporal gyrus (level: mammillary body) and parietal region (level: 1 cm behind the splenium), caudate nucleus, putamen, GP, STN, substantia nigra (SN; level: emergence of the third cranial nerve), pontine base, including the pontine nuclei, cerebellar dentate nucleus and cerebellar white matter. The posterior frontal white matter was omitted from the analysis as the quantity was very small in some cases due to variability of routine sampling. In each region, the images of 10 random microscopic fields using a ×20 objective were captured by a colour digital camera connected to the microscope (Nikon Microphot-FXA and Digit sight DS-L1) and processed with an image analysis software (Image-Pro; Media Cybernetics, Inc., Roper Industries, Rockville, USA), converted to grey-scale images and labelling was measured in pixels. Threshold was adjusted to capture the two-dimensional area of all tau-positive lesions and the same threshold setting was used throughout the study. 'Areal fraction', defined by a ratio of the tau-positive immunoreactive pixels to the total number of pixels of the whole field was computed by Image Pro and tau load for each region, that is, 'regional' tau load was expressed as percentage (areal fraction × 100%) [32]. 'Total' tau load was the sum of tau load in all 15 regions. 'Cortical' tau load was the sum of tau load in seven regions, comprised of both grey and subcortical white matter in the anterior frontal, temporal and parietal regions and grey matter in the posterior frontal region. 'Basal ganglia' tau load was the sum of tau load in four structures: caudate nucleus, putamen, GP and STN.

Quantification of tau-positive cellular lesions

The different tau-positive cellular lesions were quantified individually in 10 random fields of three selected regions (posterior frontal cortex, anterior frontal cortex and caudate), where differences in regional tau load were found to be the most robust between PSP-RS and PSP-CBS. NFTs, PreTs, TAs and CBs were individually counted. NT pathology was quantified using a four-tiered semi-quantitative grading scale (0–3, with grade 0 = no NT to grade 3 = most severe NT).

Neuronal loss in the subthalamic nucleus and substantia nigra

Neuronal loss in STN and SN were determined using a four-tiered semi-quantitative grading system by a neuropathologist (T. R.), blinded to the clinical features (0–3,

with grade 0 = no neuronal loss to grade 3 = most severe neuronal loss). SN was divided into five regions (medial, dorsomedial, dorsolateral, ventrolateral and lateral).

Corticospinal tract involvement

Microglial pathology of the corticospinal tract (CST) identified in the midbrain cerebral peduncles was assessed using CD68 immunohistochemistry by two neuropathologists (T. R. and J. H.) blinded to the clinical features. A semi-quantitative grade was established by consensus (grade 0 = baseline microglial population to grade 3 = most severe microglial pathology).

Tau biochemistry

Frontal cerebral cortex was used for tau biochemistry in two PSP-CBS, two PSP-RS cases and two pathologically diagnosed CBD cases with classical CBS presentation (CBD-CBS), which were randomly selected. Regional variation of phosphorylated tau species in PSP brains was previously reported [33]. However, tau protein extraction was limited to the frontal cortex in the present study.

Sarkosyl-insoluble tau isolation Isolation of sarkosyl-insoluble tau was carried out as previously described [34,35]. Brain tissue was homogenized in 10× volume (v/w) homogenization buffer (10 mM Tris–HCl pH 7.4, 0.8 M NaCl, 1 mM EGTA and 10% sucrose containing Complete protease inhibitor cocktail (Roche, Burgess Hill, UK). The suspension was then spun at 20 000 **g** for 20 min at 4°C and the supernatant set aside. The pellet was re-suspended in 5× volumes of homogenization buffer and re-centrifuged as above. The supernatants were combined and *N-lauryl* sarcosinate added to a concentration of 1% (w/v), and incubated at room temperature for 1 h with shaking. The mixture was then centrifuged at 100 000 **g** for 1 h at 4°C. The sarkosyl-insoluble pellet was re-suspended in 50 mM Tris–HCl pH 7.5 at 0.2 ml/g of starting material.

SDS-PAGE Sarkosyl-insoluble tau was separated on 10% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes using standard procedures. The blots were probed with a pan-tau rabbit polyclonal TP70 antibody that recognizes the carboxy-terminus of tau [36,37] (1/15 000; kind gift from Dr Diane Hanger, King's College, London) and IRDye 800CW Donkey Anti-Rabbit secondary antibody (Li-Cor Biosciences) followed by imaging on a Li-Cor Odyssey Infrared Scanner.

Haplotype analysis of the *MAPT* gene

Haplotype was determined by PCR (polymerase chain reaction) typing of the 238 bp *MAPT* H2 deletion in intron nine in 17 cases (8 PSP-CBS, 9 PSP-RS) where frozen tissue was available for DNA extraction [38,39].

Statistical analysis

The Mann–Whitney *U*-Test was used to compare tau load between PSP-CBS and PSP-RS. The null hypothesis (H0) was rejected if the *P* value was <0.05 when 'total', 'cortical' and 'basal ganglia' tau load was assessed. For 'regional' tau load assessment, *P* value of 0.0033 (0.05/15) was used to adjust for multiple comparisons; for tau-positive cellular lesion load, *P* value of 0.01 (5 different types of tau lesions: 0.05/5) was used. χ^2/Fisher's exact test or the Student's *t*-test was used to compare semi-quantitative grading or clinical data using *P* value of 0.05. The intra-rater repeatability was assessed by repeating tau quantification in four randomly selected cases (20%). The intraclass correlation coefficient was 0.80 ($P < 0.001$), indicating that the 'regional' tau load results were highly repeatable. The SPSS 17.0 program (IBM Corporation, New York, USA) was used for statistical analysis.

Results

Clinical features

PSP-CBS (Tables 1a and 2) All patients had been diagnosed with CBS/CBD by neurologists during life. Mean duration of first symptom onset to the final clinical diagnosis was 3.4 years. All cases had strikingly asymmetrical clinical features throughout the entire disease course; 10 had ideomotor limb apraxia, eight had hand dystonia, five had focal distal myoclonus, three had an alien limb phenomenon, three had non-fluent aphasia, three had cortical sensory loss and wo had hemisensory neglect. Delayed initiation of horizontal saccades was observed in three patients, two of whom also had head thrust at saccadic initiation (cases 2 & 5).

Seven patients developed ocular features suggestive of PSP including slow vertical saccades or VSGP but in most

Table 1a. Demographic, clinical and genetic haplotype data of PSP-CBS patients

	PSP-CBS									
Case no.	1	2	3	4	5	6	7	8	9	10
Gender	M	F	M	M	M	F	F	F	M	F
Age at onset (yr)	55	63.8	60.4	60.5	79.3	66.3	60	64	77	73
Age at death (yr)	64.3	70.2	66.3	68.8	82.8	77.9	70.8	72.5	81	79.2
Disease duration (yr)	9.3	6.4	5.9	8.3	3.5	11.6	10.8	8.5	4	6.2
Initial clinical Dx	PD	CBS	C.Spond.	CVD	PD	Depression	CBS	PSP	PSP	CBS
Final clinical Dx	CBS	CBS	CBS	CBS	CBS	CBS	CBS	CBS	CBS	CBS
Duration from onset to final Dx (yr)	6.3	3.5	5.2	2.1	1.5	2.6	7	6	4	3
Initial symptom(s)	Balance difficulty	Clumsy useless arm	Clumsy useless arm	Jerky arm	Falls	Gait difficulty & cognitive slowing	Clumsy useless arm & balance difficulty	Falls	Balance difficulty & slurred speech	Clumsy useless arm & falls
Asymmetrical features	+	+	+	+	+	+	+	+	+	+
Limb apraxia	+	+	+	+	+	+	+	+	+	+
Alien limb	–	+	–	–	–	–	–	–	+	+
Cortical sensory loss	–	+	+	–	–	–	–	–	–	–
Hemi-neglect	–	+	–	–	–	–	–	+	–	–
Aphasia	–	+	–	–	–	+	–	–	+	–
Hand dystonia	+	+	+	+	–	–	+	+	+	+
Clenched fist	+	+	+	+	–	–	+	–	+	+
Myoclonus	–	–	+	+	+	–	+	–	+	–
Tremor	–	–	–	–	+	–	–	–	+	–
Delayed initiation of saccades	NK	+	NK	+	+	NK	NK	–	–	NK
Slow vertical saccades	NK	–	NK	+	+	+	NK	+	NK	+
VSGP	+	–	NK	–	+	+	NK	+	+	–
Postural instability/falls within 1st yr	–	+	–	–	+	+	–	+	+	+
Cognitive decline	–	+	–	+	+	+	–	+	–	+
Personality change/apathy	–	–	–	+	–	+	–	–	–	–
Pyramidal signs	+	+	–	+	+	–	+	–	–	–
Akinetic rigidity in first 2 yrs	+	–	–	–	+	+	–	+	+	+
Dysarthria in first 2 yrs	+	–	–	+	–	–	–	–	+	–
Dysphagia in first 2 yrs	–	–	–	NK	–	–	NK	NK	+	–
Levodopa response	–	–	–	–	Mild	–	–	–	–	Mild
H1/H2 Haplotype	H1/H1	H1/H1	H1/H1	NK	H1/H1	H1/H2	H1/H1	H1/H1	NK	H1/H2

Table 1b. Demographic, clinical and genetic haplotype data of PSP-RS patients

	PSP-RS									
Case no.	11	12	13	14	15	16	17	18	19	20
Gender	F	F	M	M	F	M	M	F	F	M
Age at onset (yr)	62	65.2	63	74.3	66	61	52.1	67	72	76
Age at death (yr)	69.8	71.3	69.5	79.5	81.7	78.3	61.3	73	79.1	80.7
Disease duration (yr)	7.8	6.1	6.5	5.2	15.7	17.3	9.2	6	7.1	4.7
Initial clinical Dx	Depression	PD	Depression	PD	PD	CVD	PSP	PSP	PSP	PSP
Final clinical Dx	PSP	PSP	PSP	PSP	PSP	PSP	PSP	PSP	PSP	PSP
Duration from onset to final Dx (yr)	4	4	2.5	3	7	2.2	3	3	3	2
Initial symptom(s)	Falls & cognitive slowing	Falls	Falls & cognitive slowing	Falls	Slow up	Slurred speech	Balance difficulty	Falls	Falls	Falls
Asymmetrical features	–	–	–	–	–	–	–	–	–	–
Limb apraxia	–	–	–	–	–	–	–	–	–	–
Alien limb	–	–	–	–	–	–	–	–	–	–
Cortical sensory loss	–	–	–	–	–	–	–	–	–	–
Hemineglec	–	–	–	–	–	–	–	–	–	–
Aphasia	–	–	–	–	–	–	–	–	–	–
Hand dystonia	–	–	–	–	–	–	+	–	–	–
Clenched fist	–	–	–	–	–	–	–	–	–	–
Myoclonus	–	–	–	–	–	–	–	–	–	–
Tremor	+	–	–	–	–	–	–	–	–	+
Delayed initiation of saccades	NK	–	–	NK	–	–	–	–	–	NK
Slow vertical saccades	+	NK	+	NK	+	+	NK	+	+	NK
VSGP	+	+	+	+	+	+	+	+	+	+
Postural instability/falls within 1st yr	+	+	+	+	+	+	+	+	+	+
Cognitive decline	+	–	+	–	–	–	–	+	–	–
Personality change/apathy	+	–	+	+	–	–	+	+	–	–
Pyramidal signs	–	–	–	–	–	–	–	–	–	–
Early akinetic rigidity in first 2 yrs	+	+	+	+	+	+	+	+	+	+
Early dysarthria in first 2 yrs	+	NK	–	–	–	+	+	NK	+	–
Early dysphagia in first 2 yrs	+	–	–	–	–	–	+	NK	+	–
Levodopa response	–	–	–	–	–	–	–	–	–	–
H1/H2 Haplotype	H1/H1	H1/H1	NK	H1/H1	H1/H1	H1/H1	H1/H1	H1/H1	H1/H1	H1/H1

CBS, corticobasal syndrome; C.Spond, cervical spondylosis; CVD, cerebrowascular disease; Dx, diagnosis; F, female; M, male; NA, not applicable; NK, not known; PD, Parkinson's disease; PSP, progressive supranuclear palsy, RS, Richardson's syndrome; VSGP, vertical supranuclear gaze palsy; yr, year.

Table 2. Demographic features between PSP-CBS and PSP-RS

	PSP-CBS	*PSP-RS*	P *values (Student's* t-*test)*
	(mean years ± SD)		
Mean age of symptom onset	65.9 ± 8.0	65.9 ± 7.1	0.98
Mean age of death	73.4 ± 6.4	74.4 ± 6.5	0.72
Mean disease duration	7.5 ± 2.7	8.6 ± 4.4	0.51

cases these occurred in the advanced stage of the illness. Two exceptions were cases 8 and 9 who developed VSGP within 4 years from symptom onset and an initial clinical diagnosis of PSP was considered, but was later revised to CBS after the onset of asymmetrical cortical symptoms. Six patients developed postural instability or falls within the first year of symptom onset. Nevertheless, VSGP (χ^2, $P = 0.016$) and postural instability or early falls were still more frequent in PSP-RS than in PSP-CBS. Pyramidal signs were more frequent in PSP-CBS ($n = 5$) than in PSP-RS ($n = 0$) (χ^2, $P = 0.016$). Extensor plantars and hyperreflexia were noted in five PSP-CBS patients, three of whom also had spasticity and one had pyramidal weakness, but none of these features was observed in PSP-RS.

PSP-RS (Tables 1b and 2) All PSP-RS patients had a final clinical diagnosis of probable PSP and had VSGP including downgaze abnormalities and early postural instability or falls. Three patients had cognitive decline and five had frontal type personality change characterized by apathy and abulia.

Pathological findings and clinicopathological correlations

Both PSP-CBS and PSP-RS groups met established pathological criteria of PSP [20–22]. All inclusion types were immunoreactive for 4R tau by differential immunohistochemistry but negative for 3R tau in all cases [24], which was an expected finding for PSP.

'Regional' tau load The median 'regional' tau load in the posterior frontal cortical grey matter (PSP-CBS: 0.59; PSP-RS: 0.05), anterior frontal cortical grey matter (PSP-CBS: 0.06; PSP-RS: 0.03) and parietal subcortical white matter (PSP-CBS: 0.06; PSP-RS: 0.01) was significantly greater in PSP-CBS than in PSP-RS ($P < 0.0033$ in all). The median

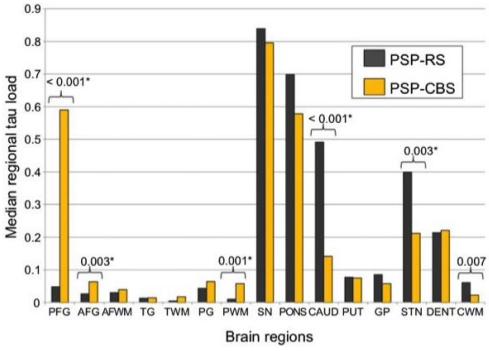

Figure 1. Quantitative data illustrating median regional tau load in PSP-RS (black) and PSP-CBS (yellow) in 15 selected regions. Error bars represent 95% confidence interval. *Represents statistical significance, $P < 0.0033$ using the Mann–Whitney *U*-Test. PFG, posterior frontal grey matter; AFG, anterior frontal grey matter; AFWM, anterior frontal white matter; TG, temporal grey matter; TWM, temporal white matter; PG, parietal grey matter; PWM, parietal white matter; SN, substantia nigra; PONS, pons; CAUD, caudate; PUT, putamen; GP, globus pallidus; STN, subthalamic nucleus; DENT, dentate nucleus; CWM, cerebellar white matter.

'regional' tau load in the caudate (PSP-CBS: 0.14; PSP-RS: 0.49; $P < 0.001$), STN (PSP-CBS: 0.21; PSP-RS: 0.40; $P < 0.001$) and cerebellar white matter (PSP-CBS: 0.02; PSP-RS: 0.06; $P = 0.007$ with borderline significance) was greater in the PSP-RS than in PSP-CBS (Figures 1 and 2).

The presence of delayed initiation of horizontal saccades in PSP-CBS had a moderate correlation with an increased total parietal 'tau load' (Spearman's correlation coefficient = 0.59; $P < 0.001$). However, other cortical features such as cortical sensory loss, alien limb phenomenon or hemi-sensory neglect did not correlate with the parietal 'tau load' ($P > 0.05$) or other 'regional tau load'.

'Total', 'cortical' and 'basal ganglia' tau load (Figure 3) There was no difference in 'total' tau load between the PSP-CBS and PSP-RS groups ($P = 0.176$, Figure 3). However, PSP-CBS had an increased 'cortical' tau load when compared with PSP-RS ($P < 0.001$); and the 'basal ganglia' tau load was greater in PSP-RS than in PSP-CBS ($P = 0.003$).

In five PSP-CBS cases, the half brains examined were contralateral to the side with the more predominant clinical symptoms and signs. The median 'total' and 'cortical' tau load were numerically, but not statistically, greater in these five cases (total tau load = 5.3; cortical tau

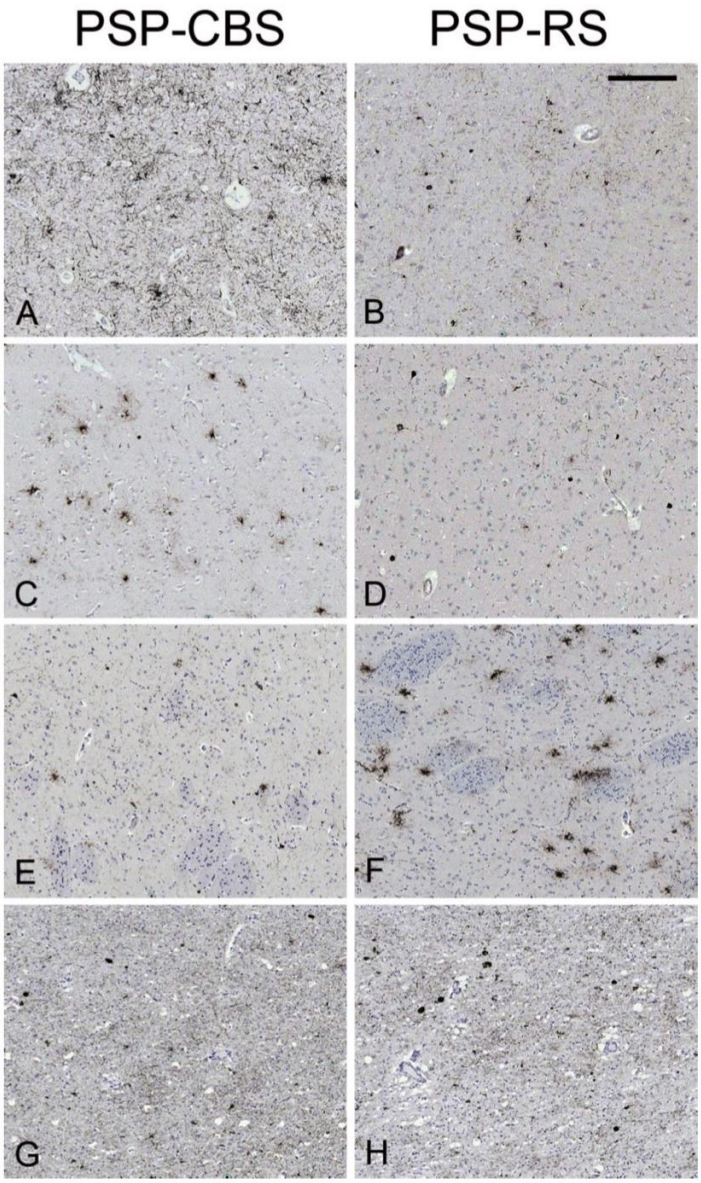

Figure 2. Tau immunohistochemistry in selected brain regions of the two most representative cases. PSP-CBS has significantly greater tau load in the posterior frontal (**A**) and anterior frontal grey matter (**C**) when compared with PSP-RS (**B**, **D**). Regional tau load in the caudate and subthalamic nucleus are greater in PSP-RS (**F**, **H**) than in PSP-CBS (**E**, **G**). Median tau load values. Posterior frontal grey matter: (**A**) PSP-CBS: 1.09; (**B**) PSP-RS: 0.05; Anterior frontal grey matter: (**C**) PSP-CBS: 0.18; (**D**) PSP-RS: 0.04; Caudate: (**E**) PSP-CBS: 0.11; (**F**) PSP-RS: 1.06; Subthalamic nucleus: (**G**) PSP-CBS: 0.05; (**H**) PSP-RS: 0.29. Tau load for each region, that is, 'regional' tau load was expressed as percentage (areal fraction × 100%); 'areal fraction', which was computed by Image Pro, was defined by a ratio of the tau-positive immunoreactive pixels to the total number of pixels of the whole field. AT8 immunohistochemistry, bar in panel **B** represents 225 microns in all the panels.

load = 1.4) compared with the remaining PSP-CBS cases (total tau load = 4.0; cortical tau load = 1.0).

Tau-positive cellular lesions In the posterior frontal cortical grey matter, all types of tau lesions were more numerous in PSP-CBS than in PSP-RS (NFTs, TAs, CBs and NTs: $P < 0.001$; PreTs: $P = 0.005$). In the anterior frontal grey matter, there were numerically, but not statistically, more NFTs, CBs and NTs in PSP-CBS than in PSP-RS ($P > 0.01$ in all). In the caudate, there were more TAs, NTs, and NFTs, in PSP-RS than in PSP-CBS (TAs and NTs: $P < 0.001$, NFTs: $P = 0.01$).

Neuronal loss In the STN, the median semi-quantitative rating score for neuronal loss was moderate (grade 2) and there was no difference between the two groups (χ^2; $P \geq 0.05$). In the SN, neuronal loss was more severe in the dorsolateral (χ^2; $P = 0.033$) and ventrolateral (χ^2; $P = 0.018$) subregions in PSP-RS than in PSP-CBS (Figure 4).

CST involvement There was a more severe microglial response in the CST in PSP-CBS, ranged from mild to severe, than in PSP-RS, in which CST involvement was very mild (χ^2; $P = 0.035$) (Figure 5).

Additional pathological findings The CERAD Aβ plaque score ranged from 'absent' to 'sparse', except for two PSP-CBS and one PSP-RS cases, which had a 'moderate' score [27]. Small vessel cerebrovascular disease was noted in one PSP-CBS and one PSP-RS case. Other additional pathological findings are summarized in Table 3.

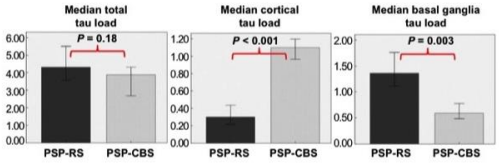

Figure 3. The median 'total tau load' between the PSP-CBS and PSP-RS groups are the same. However, the PSP-CBS group has greater median 'cortical tau load' and less 'basal ganglia tau load' than the PSP-RS group (Mann–Whitney *U*-Test). 'Cortical tau load' is the sum of regional tau load of posterior frontal grey matter, anterior frontal grey and white matter, temporal grey and white matter, parietal grey and white matter. 'Basal ganglia tau load' is the sum of regional tau load of caudate, putamen, globus pallidus and subthalamic nucleus. 'Total tau load' is the sum of regional tau load of all 15 brain regions. Error bars represent 95% confidence interval.

Tau biochemistry and haplotype analysis

Western blots of the sarkosyl-insoluble tau fractions from the frontal cortical homogenates showed the characteristic doublet at 64 and 68 kDa indicating predominant 4R tau in PSP-CBS, PSP-RS and CBD-CBS cases (Figure 6). Our PSP-CBS and PSP-RS cases showed a single band at approximately 33 kDa, whereas the CBD-CBS cases had a doublet at approximately 37 kDa (Figure 6), which are consistent with previous findings on the molecular differences in the low molecular weight proteolytic fragments between CBD and PSP [14,40]. There was no significant association between H1/H1 or H1/H2 genotype with either of the PSP subgroups (χ^2 test; $P = 0.21$, Table 1).

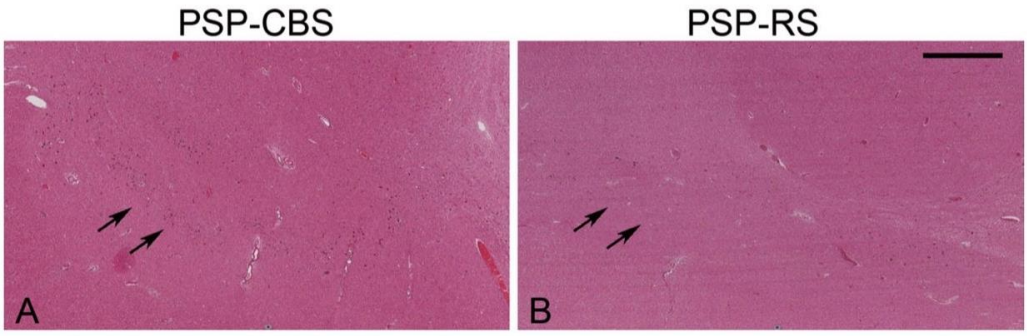

Figure 4. PSP-RS (**B**) has greater degree of neuronal loss in the ventrolateral (arrows) and dorsolateral substantia nigra than in PSP-CBS (**A**). Haematoxylin and eosin method, bar in panel **B** represents 1135 microns in both panels.

ces than in PSP-RS [12]. Here, by assessing a greater number of cases and more brain regions, we validated their findings by showing increased tau load in the cortical regions predominantly in the posterior frontal grey matter, anterior frontal grey matter and parietal white matter in PSP-CBS. We also extended the quantitative tau assessment to other non-cortical regions which enabled us to identify a reduced tau load in the caudate, STN and cerebellar white matter in the CBS variant. It is noteworthy that the increased cortical tau load is compensated by the reduced basal ganglia tau load in PSP-CBS resulting in the total tau load, determined as the sum of the all regional tau load, being similar in the two PSP groups.

Increased cortical tau pathology has also been documented in PSP variants with cortical features including 'atypical' PSP with progressive apraxia of speech and non-fluent aphasia [42], PSP-bvFTD [11,43], and, together with PSP-CBS, these clinical phenotypes are collectively referred as the 'cortical' PSP variants [44]. On the other hand, PSP-P and PSP-PAGF, which are considered as the 'brainstem' variants of PSP, have less severe overall tau pathology when compared with PSP-RS [5,6]. Interestingly, these 'brainstem' variants are associated with a more benign disease course and a longer disease duration compared with the classical PSP-RS [5,6,30]; whereas the disease duration of our PSP-CBS group was similar to that of PSP-RS group previously reported [2]. We speculate that the total tau pathology may inversely correlate with the disease duration in PSP variants and while the 'brainstem' PSP variant appears to be a more 'benign' form of PSP, the 'cortical' PSP variant represents a deviation from the classical presentation determined by a shift of tau pathology from the basal ganglia to the cerebral cortex. By selecting only cases with limited Alzheimer-type NFT pathology and assessing coexisting secondary pathologies, it is clear that the clinical presentation in our PSP-CBS cases was closely associated with the topographical severity of tau pathology which could not otherwise be explained by secondary pathologies. We also demonstrated that the regional differences in tau load between the two PSP groups were contributed by neuronal and glial lesions characteristic of PSP pathology rather than Alzheimer-related tau pathology [45].

A recent detailed clinicopathological study from the Mayo Clinic compared the characteristics of CBS and RS clinical phenotypes in pathologically confirmed CBD cases [14]. Their study on CBD also demonstrated significant differences in the topographical severity of tau pathology between the two CBD subtypes, which correlated with the different clinical presentations. Similar to the findings in our PSP-CBS cases, their study showed that the CBD-CBS cases had more severe tau deposition in the cortical regions and less severe tau pathology in the lower brainstem and cerebellum when compared with the CBD-RS cases. However, total tau load and the contribution by different neuronal and glial lesions to the tau pathology were not assessed [14].

The STN is one of the regions characteristically targeted by the PSP disease process [20,46] while this nucleus is better preserved in cases with pathologically confirmed CBD. In our PSP-CBS cases, the atrophy and neuronal loss in the STN was as severe as in the PSP-RS cases, despite the regional tau load of the STN being less in PSP-CBS than in PSP-RS, indicating that glial, rather than neuronal tau, might have significantly contributed to the differences in tau load. It is noteworthy that a relatively milder tau pathology in the STN has been documented in other PSP variants such as 'atypical' PSP with progressive apraxia of speech and non-fluent aphasia [42].

In PSP, cell loss in the SN affects both the pigmented neurones of the pars compacta and non-pigmented neurones in the pars reticulata, whereas neurones in the medial nigra are relatively preserved [47]. In the present study, neuronal loss was less severe in the ventrolateral and dorsolateral subregions in PSP-CBS when compared with PSP-RS (Figure 4). This regional difference may, in part, influence the clinical features due to the resulting selective damage to the dopaminergic and GABAergic neuronal nigral projections [47].

Pyramidal signs were documented in half of our PSP-CBS cases, but they were absent in our PSP-RS cohort. Pyramidal signs are relatively uncommon in PSP and in one series they were present in only one fifth of all pathologically confirmed PSP cases [2]. On the other hand, 60% of pathologically confirmed CBD cases had pyramidal signs [48]. In CBD, the pathological involvement of the primary motor cortex including loss of Betz cells is a common finding, explaining the presence of pyramidal signs [44,48]. The common occurrence of pyramidal signs in our PSP-CBS cohort can be explained by the abundant tau pathology in the primary motor cortex, which was 12-fold greater in PSP-CBS than in PSP-RS. There was also more severe microglial pathology in the CST in PSP-CBS than in PSP-RS. CST degeneration and significant tau pathology in the motor cortex are also prominent in a group of 'atypical' PSP cases reported in the literature which sometimes clini-

ces than in PSP-RS [12]. Here, by assessing a greater number of cases and more brain regions, we validated their findings by showing increased tau load in the cortical regions predominantly in the posterior frontal grey matter, anterior frontal grey matter and parietal white matter in PSP-CBS. We also extended the quantitative tau assessment to other non-cortical regions which enabled us to identify a reduced tau load in the caudate, STN and cerebellar white matter in the CBS variant. It is noteworthy that the increased cortical tau load is compensated by the reduced basal ganglia tau load in PSP-CBS resulting in the total tau load, determined as the sum of the all regional tau load, being similar in the two PSP groups.

Increased cortical tau pathology has also been documented in PSP variants with cortical features including 'atypical' PSP with progressive apraxia of speech and non-fluent aphasia [42], PSP-bvFTD [11,43], and, together with PSP-CBS, these clinical phenotypes are collectively referred as the 'cortical' PSP variants [44]. On the other hand, PSP-P and PSP-PAGF, which are considered as the 'brainstem' variants of PSP, have less severe overall tau pathology when compared with PSP-RS [5,6]. Interestingly, these 'brainstem' variants are associated with a more benign disease course and a longer disease duration compared with the classical PSP-RS [5,6,30]; whereas the disease duration of our PSP-CBS group was similar to that of PSP-RS group previously reported [2]. We speculate that the total tau pathology may inversely correlate with the disease duration in PSP variants and while the 'brainstem' PSP variant appears to be a more 'benign' form of PSP, the 'cortical' PSP variant represents a deviation from the classical presentation determined by a shift of tau pathology from the basal ganglia to the cerebral cortex. By selecting only cases with limited Alzheimer-type NFT pathology and assessing coexisting secondary pathologies, it is clear that the clinical presentation in our PSP-CBS cases was closely associated with the topographical severity of tau pathology which could not otherwise be explained by secondary pathologies. We also demonstrated that the regional differences in tau load between the two PSP groups were contributed by neuronal and glial lesions characteristic of PSP pathology rather than Alzheimer-related tau pathology [45].

A recent detailed clinicopathological study from the Mayo Clinic compared the characteristics of CBS and RS clinical phenotypes in pathologically confirmed CBD cases [14]. Their study on CBD also demonstrated significant differences in the topographical severity of tau pathology between the two CBD subtypes, which correlated with the different clinical presentations. Similar to the findings in our PSP-CBS cases, their study showed that the CBD-CBS cases had more severe tau deposition in the cortical regions and less severe tau pathology in the lower brainstem and cerebellum when compared with the CBD-RS cases. However, total tau load and the contribution by different neuronal and glial lesions to the tau pathology were not assessed [14].

The STN is one of the regions characteristically targeted by the PSP disease process [20,46] while this nucleus is better preserved in cases with pathologically confirmed CBD. In our PSP-CBS cases, the atrophy and neuronal loss in the STN was as severe as in the PSP-RS cases, despite the regional tau load of the STN being less in PSP-CBS than in PSP-RS, indicating that glial, rather than neuronal tau, might have significantly contributed to the differences in tau load. It is noteworthy that a relatively milder tau pathology in the STN has been documented in other PSP variants such as 'atypical' PSP with progressive apraxia of speech and non-fluent aphasia [42].

In PSP, cell loss in the SN affects both the pigmented neurones of the pars compacta and non-pigmented neurones in the pars reticulata, whereas neurones in the medial nigra are relatively preserved [47]. In the present study, neuronal loss was less severe in the ventrolateral and dorsolateral subregions in PSP-CBS when compared with PSP-RS (Figure 4). This regional difference may, in part, influence the clinical features due to the resulting selective damage to the dopaminergic and GABAergic neuronal nigral projections [47].

Pyramidal signs were documented in half of our PSP-CBS cases, but they were absent in our PSP-RS cohort. Pyramidal signs are relatively uncommon in PSP and in one series they were present in only one fifth of all pathologically confirmed PSP cases [2]. On the other hand, 60% of pathologically confirmed CBD cases had pyramidal signs [48]. In CBD, the pathological involvement of the primary motor cortex including loss of Betz cells is a common finding, explaining the presence of pyramidal signs [44,48]. The common occurrence of pyramidal signs in our PSP-CBS cohort can be explained by the abundant tau pathology in the primary motor cortex, which was 12-fold greater in PSP-CBS than in PSP-RS. There was also more severe microglial pathology in the CST in PSP-CBS than in PSP-RS. CST degeneration and significant tau pathology in the motor cortex are also prominent in a group of 'atypical' PSP cases reported in the literature which sometimes clini-

cally present with CBS [44,49]; however, whether these cases should be classified as PSP has recently been questioned [50].

All PSP-CBS cases had received a final clinical diagnosis of CBS and they all had markedly asymmetrical cortical and extrapyramidal features, including unilateral limb clumsiness with a progressively maladroit and functionally useless hand. In the past, the presence of marked asymmetrical clinical signs would exclude the clinical diagnosis of PSP, but this concept has been challenged in recent years with the findings of clinicopathological series confirming asymmetrical presentations in some PSP variants [2,17]. Previously, a *post mortem* report of a Japanese patient who had focal limb dystonia and levitation revealed significantly more tau pathologies in the frontal cortices, basal ganglia and brain stem in the contralateral half brain than the ipsilateral half brain [51]. In our PSP-CBS cohort, there was numerically greater total tau load and cortical tau load in five cases where the contralateral half brain was available for evaluation when compared with the other five cases where ipsilateral half brain was examined. However, we cannot conclude if the tau load is greater in the clinically more manifested hemisphere within an individual as only half brains were used. Asymmetrical limb apraxia and delayed initiation of horizontal saccades are clinical features suggestive of underlying parietal lobe dysfunction and are characteristic features of CBS [52]. We found that regional tau load in the parietal white matter was 5-fold greater in PSP-CBS than in PSP-RS and that PSP-CBS patients who had delayed initiation of horizontal saccades also had greater regional tau load in the parietal cortex and white matter.

Three patients had delayed initiation of horizontal saccades and, interestingly, half of them also had VSGP in the late stage of the illness, with involvement of downgaze, a diagnostic prerequisite for the diagnosis of PSP-RS [53,54]. VSGP is rare in CBD with classical CBS presentation (CBD-CBS), and was noted in only 18% of cases in the Mayo Clinic series [14] and was not observed in the QSBB series [17]. Six PSP-CBS patients had recurrent falls in the first year of their illness, whereas early falls were less frequent in CBD-CBS cases and were recorded in only 20% and 18% in the QSBB and Mayo Clinic series, respectively [14,17]. We postulate that early postural instability, falls and supranuclear downgaze palsy in patients with CBS are clinical clues which when present, suggest an underlying PSP pathology even though there are also signs of CBS. Nevertheless, three cases in our PSP-CBS group (cases 2, 7, 10) had a pure CBS presentation throughout the disease course and did not have any tell-tale signs of PSP. This is in concordance with our experience based on clinicopathological evaluation of cases in the QSBB that some pathologically confirmed PSP and CBD cases present with a pure clinical syndrome such as CBS or RS irrespective of the underlying pathology, whereas some cases manifest overlap clinical features such as RS or CBS at the same time and occasionally, the clinical syndromes temporally evolve from one to another throughout the disease course as previously described by Kertesz *et al.* [9].

Data from transgenic animal studies indicate that soluble rather than fully aggregated tau species may ultimately be responsible for neuronal degeneration and cell death [55]. However, the findings in this study support the notion that neuronal and glial inclusions composed of fibrillar pathological tau are useful and clinically valid pathological markers of the underlying neurodegenerative process. We have provided comprehensive evidence that the topographical severity of tau pathology in PSP is closely associated with its clinical manifestation [5,6,11,30,42]. This is comparable to the findings in Alzheimer's disease where cognitive deficit shows a far better correlation with tau lesions than with Aβ plaques [56] as well as in other primary tauopathies [44,57]. A better understanding of the factors that influence the selective pathological vulnerability in different PSP variants will provide further insights into the neurodegenerative process underlying tauopathies.

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Authors' roles

1. Research project: A, Conception; B, Organization; C, Execution.
2. Statistical analysis: A, Design; B, Execution; C, Review and Critique.
3. Manuscript: A, Writing of the first draft; B, Review and Critique.

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Astrogliopathy predominates the earliest stage of corticobasal degeneration pathology

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Animal models have shown that tau seeding and propagation are strain- and neural network-specific. The study of preclinical cases is valuable to gain insights into early pathological features of corticobasal degeneration and its progression. Three preclinical corticobasal degeneration cases and six age-matched end-stage corticobasal degeneration cases were included in this study. Tau immunohistochemistry performed in 20 brain regions and quantitative assessment of regional tau load using image analysis were performed. Semi-quantitative grading of tau-positive cellular lesions and neuronal loss in the frontal, parietal and temporal cortices, striatum, substantia nigra and subthalamic nucleus were assessed. All preclinical cases were clinically asymptomatic but had widespread tau lesions in the typically affected regions in corticobasal degeneration and the pathognomonic astrocytic plaques were the most prominent lesion type in the anterior frontal and striatal regions. Mean total tau load (sum of all regional tau load) of end-stage corticobasal degeneration cases were nine times greater than that of the preclinical cases ($P = 0.04$) and less tau load was found in all regions of the preclinical cases. An anterior-to-posterior tau load ratio in the frontal cortex in preclinical cases was 12-fold greater than in end-stage corticobasal degeneration cases. Relatively greater tau burden in the anterior frontal cortex, striatum and subthalamic nucleus suggests the striatal afferent connection to the dorsolateral prefrontal cortex and basal ganglia circuitry are the earliest neural network connections affected by corticobasal degeneration-related tau pathology. Differential distribution of the tau pathology to selective cortical regions in these preclinical cases implies phenotypic presentation may be predetermined at a very early stage of the disease process. Neuronal loss of the substantia nigra was either absent or very mild in the preclinical cases and was moderate to severe in end-stage corticobasal degeneration cases ($P < 0.05$). Our findings suggest that a threshold of pathological burden in the 'right' anatomical regions needs to be reached before the onset of clinical symptoms. The early prominence of the astrocytic plaques in relation to sparse neuronal lesions leads one to speculate that corticobasal degeneration may begin as an astrogliopathy at a very early disease stage but neuronal lesions gradually take over as the predominant lesion type in advanced disease.

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Abbreviations: CBD = corticobasal degeneration, CBS = corticobasal syndrome; FTD = frontotemporal dementia; PSP = progressive supranuclear palsy; RS = Richardson syndrome

Introduction

Corticobasal degeneration (CBD) is a progressive neurodegenerative tauopathy characterized by the accumulation of hyperphosphorylated 4-repeat tau in the neurons and glia in both cortical and subcortical regions (Dickson *et al.*, 2002). Astrocytic plaques are pathognomonic for CBD and are numerous in affected cerebral cortical areas and the striatum. Neuropil threads are usually numerous and, along with neurofibrillary tangles, pretangles and oligodendroglial coiled bodies, are widespread but are generally greater in the cortex and basal ganglia than in the brainstem and cerebellum. αB-crystallin-immunoreactive ballooned neurons are common and support the diagnosis of CBD (Fig. 1). They are observed in the cortical regions, more frequently in the superior frontal gyrus. In affected cortical areas, spongiosis is usually most evident in layers 2 and 3 and astrogliosis is prominent at the grey–white matter junction with myelin loss and microgliosis in the cerebral white matter. Loss of pigmented cells and gliosis in the substantia nigra are consistent findings.

Similar to progressive supranuclear palsy (PSP) (Ling *et al.*, 2013), which is also a 4-repeat tauopathy, the distribution of neuronal loss and severity of tau pathology in CBD closely correlate with its heterogeneous clinical presentations (Kouri *et al.*, 2011). Corticobasal syndrome (CBS) is the classic presentation with asymmetrical focal cortical signs including limb apraxia, dystonia and akinetic-rigidity (CBD-CBS) (Rebeiz *et al.*, 1967, 1968). Clinicopathological studies have shown that CBD is also associated with other phenotypic presentations such as Richardson syndrome (CBD-RS; i.e. a PSP-like syndrome), behavioural variant frontotemporal dementia (CBD-bvFTD) or primary progressive aphasia (CBD-PPA) (Ling *et al.*, 2010). Clinical criteria have been proposed to capture the different clinical phenotypes caused by underlying CBD pathology (Armstrong *et al.*, 2013). MRI studies showed that cortical atrophy in CBD-CBS was most marked in the posterior half of the frontal lobe with the superior frontal gyrus being often more affected than the middle and inferior frontal gyri (Whitwell *et al.*, 2010). In such cases, the pre- and postcentral regions are also affected to varying degrees, but the temporal and occipital lobes are usually spared. In CBD-bvFTD and CBD-PPA, atrophy is more severe in the frontal and temporal lobes (Whitwell and Josephs, 2012).

Hierarchical staging schemes depicting stereotypic spatiotemporal progression of neuronal vulnerability as the disease progresses have been proposed for conditions with tau pathology such as Alzheimer's disease (Braak *et al.*, 2006), argyrophilic grain disease (Saito *et al.*, 2004), Pick's disease (Irwin *et al.*, 2016) and chronic traumatic encephalopathy (McKee *et al.*, 2013) as well as for Parkinson's disease (Braak *et al.*, 2003), a synucleinopathy, bvFTD (Brettschneider *et al.*, 2014) and amyotrophic lateral sclerosis (Brettschneider *et al.*, 2013) with phosphorylated

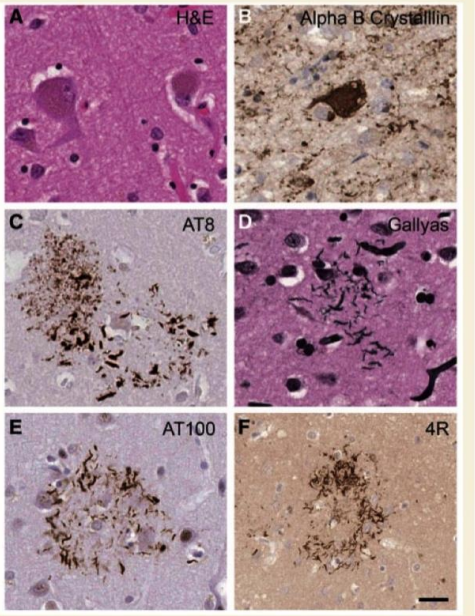

Figure 1 Ballooned neurons (A and B; end-stage CBD Case 6) and astrocytic plaques (C–F; preclinical CBD Case 1). Ballooned neuron is absent in Cases 1 and 2. Occasional ballooned neurons observed in the cingulate cortex in Case 3 are probably associated with the co-existing argyrophilic grain disease rather than CBD pathology. Astrocytic plaque is a prominent histological finding in preclinical CBD cases, especially in the anterior frontal cortex and caudate, and is immunoreactive for AT8 (**C**), AT100 (**E**) and 4-repeat tau (**F**), but not 3-repeat tau, and is Gallyas-positive (**D**). Scale bar = 10 μm.

43-kDa TAR DNA-binding protein (TDP-43) pathology. Animal models and functional MRI studies suggested that the progression of tau pathology in conditions such as Alzheimer's disease, CBD and PSP are strain- and neural network-specific (Clavaguera *et al.*, 2009, 2013; Niethammer *et al.*, 2014; Piattella *et al.*, 2015; Ahmed *et al.*, 2016). In recent years, the concepts of tau seeding, cell-to-cell and region-to-region 'spread' of tau pathology, analogous to the prion-like conformational templating mechanism, have been proposed as the mechanism responsible for the progression in these neurodegenerative conditions (Lewis and Dickson, 2016). The study of preclinical cases provides a unique resource to detect early pathological changes of the neurodegenerative condition. Similar concepts have been proposed in incidental Lewy body disease representing either preclinical Parkinson's disease (Dickson *et al.*, 2008) or dementia with Lewy bodies (Frigerio *et al.*, 2011) and in preclinical Alzheimer's disease (Braak *et al.*, 2011). In CBD, the regions affected early by tau pathology and its subsequent spatiotemporal progression are not known. This study quantitatively analysed the CBD pathological changes in three preclinical cases and compared them with end-stage CBD cases with an aim to determine the early pathological features of CBD.

Materials and methods

Case material

As part of a large-scale study on CBD, we collected 120 CBD cases from 12 UK, European and USA centres. Of these, three cases were identified to have preclinical CBD pathology. These cases had the pathological hallmarks of CBD but were clinically asymptomatic. Six age-matched end-stage CBD cases were selected as controls, three of which had the clinical phenotype of CBS (CBD-CBS) and three clinically presented with Richardson syndrome (CBD-RS). All six end-stage CBD cases had clinically advanced disease following progressive deterioration and died of end-stage disease; their post-mortem findings fulfilled the pathological diagnostic criteria for CBD (Dickson *et al.*, 2002). End-stage CBD cases with minimal concurrent Alzheimer-type and vascular pathologies were selected. Histological examination and diagnostic confirmation were established in all cases by a neuropathologist (T.R.). This Queen Square Brain Bank study was approved by a London Multi-Centre Research Ethics Committee and tissue is stored for research under a license from the Human Tissue Authority. Clinical information was extracted from all available medical records by a neurologist (H.L.).

Neuropathological methods

For Cases 2 and 3, tissue slides were requested from Taub Institute for Research on Alzheimer's disease and the Aging Brain, Columbia University Medical Center, New York, USA and the Institute of Neurology, Medical University of Vienna, Austria, respectively. For Case 1 (from Queen Square Brain Bank) and all six end-stage CBD cases, the brains were divided in the mid-sagittal plane. One half, chosen randomly, was frozen, and the other half was immersed and fixed in 10% buffered formalin for 3 weeks before neuropathological examination. Tissue blocks were taken using the Queen Square Brain Bank protocol. Histological sections (8-μm thick) were stained using routine histological (haematoxylin and eosin) and silver staining (Gallyas) (Braak *et al.*, 2011) techniques. Immunohistochemistry with antibodies to the following antigens: tau (AT8 clone; Thermo scientific MN1020; 1:600), 3-repeat tau (gift from Dr Rohan de Silva; 1:150) and 4-repeat tau (gift from Dr Rohan de Silva; 1:750), AT100 (Thermo Scientific MN1060; 1:200), αB-crystallin (Leica Biosystems NCL-ABCrys-512, clone G2JF; 1:300), amyloid-β peptide (Biosource international, Camarillo, CA, Mouse Dako, clone 6F/3D; 1:100), transactive response DNA-binding protein 43 kDa (TDP-43; monoclonal; clone 2E2-D3; 1:2000), p62 (BD Transduction Labs, 1:200) and α-synuclein (Novocastra; 1:50) was performed using a standard avidin-biotin method. The following additional pathologies were systematically assessed: p62-positive neuronal cytoplasmic inclusions seen in cases with *C9orf72* mutation, cerebral amyloid angiopathy, argyrophilic grain disease (Saito *et al.*, 2004) and TDP-43 proteinopathy. For determining the level of Alzheimer's disease neuropathological change, ABC score were established according to the National Institute on Aging-Alzheimer's Association (NIA-AA) Guidelines (Hyman *et al.*, 2012).

Quantitative analysis of tau load

Twenty brain regions which are known to be affected in CBD and whose involvement is predicted to contribute to the clinical features were selected: the anterior frontal cortex [Brodmann area (BA) 9 or prefrontal cortex] grey matter and white matter, posterior frontal cortex (BA 4 or primary motor cortex) grey and white matter, middle temporal gyrus (BA 21) grey and white matter, superior parietal lobule (BA 7) grey and white matter, hippocampal formation (CA1–4, granular cell layer of the dentate gyrus, subiculum), amygdala, caudate, putamen, globus pallidus, subthalamic nucleus, midbrain tectum and tegmentum, pontine tegmentum and base, cerebellar dentate nucleus and white matter. The posterior frontal cortex and the subthalamic nucleus were not available in Case 2.

Histological AT8-stained slides were digitized on a Leica SCN400F scanner with a ×20 objective. Slides were viewed and managed on Leica Slidepath. Brain regions of interest were manually selected and digitally outlined (H.L. and K.D.) using Definiens Developer 2.3. Threshold was adjusted to capture the 2D area of all AT8-stained lesions (brown) and the same threshold setting was used for all cases. For each selected region, the 'areal fraction', defined by the ratio of the total area occupied by the tau-immunoreactive lesions and the entire area of interest, was computed by Definiens Developer 2.3. 'Regional' tau load for each brain region was expressed as percentage (areal fraction × 100) (Gundersen *et al.*, 1988). 'Total' tau load was the sum of tau load in all 20 regions. 'Cortical' tau load was the sum of tau load of grey and white matter of the anterior frontal, posterior frontal, temporal and parietal regions. 'Basal ganglia' tau load was the sum of tau load of the caudate, putamen, globus pallidus and subthalamic nucleus.

Quantitative analysis of cellular lesion types

Tau-positive cellular lesions including neuronal lesions (neurofibrillary tangles and pretangles), astrocytic plaques and coiled bodies were manually counted (by H.L.), while neuropil threads were graded semiquantitatively using a four-tier scale (0–3 with grade 0 = absent to grade 3 = severe) at × 20 objective in five random fields (three random fields in the substantia nigra, subthalamic nucleus and cerebellar dentate nucleus due to their relatively small regional areas) in selected brain regions. A mean score for each lesion type was generated for each brain region. Semiquantitative analysis of different cellular lesion types using a four-tier scale was performed in the hippocampal formation.

Neuronal loss in the substantia nigra

Neuronal loss in the substantia nigra was determined using a four-tier semiquantitative grading system by a neuropathologist (T.R.): 0–3 with grade 0 = no neuronal loss to grade 3 = most severe neuronal loss. The substantia nigra was divided for the grading assessment into five subregions: medial, dorsomedial, dorsolateral, ventrolateral and lateral.

MAPT gene sequencing

DNA was extracted from the frozen brain tissue of the three preclinical CBD cases. Exons 10-13 of the *MAPT* gene were screened through Sanger sequencing for known pathological mutations. MAPT haplotypes were determined through the H1/H2-tagging SNP rs1052553 (Pittman *et al.*, 2005).

Statistical analysis

The SPSS 24.0 statistical package (IBM Corporation, New York, USA) was used. Log transformation was performed to normalize data where indicated including regional, total, cortical and basal ganglia tau load. Student's *t*-test and ANOVA were used to compare mean tau load ($\log_{10}$), and continuous demographic data. Pearson χ^2 test was used to compare the neuronal-to-astrocytic lesion ratios between the two CBD groups. Multiple regression analysis was performed to study which of the cellular lesion types best correlate with tau load in the cortical regions. *P*-value of 0.05 was used. Corrections for multiple comparisons of mean regional tau load were performed using a *P*-value of 0.0025 (20 regions of interest). However, due to the small sample size of the preclinical cases, results without adjustment for multiple comparisons are also reported and discussed.

Results

Overview

The three preclinical CBD cases (Cases 1–3) were clinically asymptomatic for any progressive neurodegenerative disorder and incidental findings of astrocytic plaques, the histological hallmark of CBD (Fig. 1) and a combination of neuronal and glial tau pathologies were identified at post-mortem. Six end-stage CBD cases were selected as age-matched controls and were made up of two different clinical phenotypes, CBD-CBS ($n = 3$) and CBD-RS ($n = 3$).

The mean age at death of the preclinical CBD group was 76.0 years [standard deviation (SD) = 13.0], whereas the mean age at death of the six end-stage CBD cases and our large end-stage CBD cohort collected from 12 international centres for an ongoing pathological staging study was 70.2 years (SD = 5.2 versus preclinical CBD cases: $P = 0.35$) and 70.6 years ($n = 109$, SD = 7.9, range = 48 to 88 years, versus preclinical CBD cases: $P = 0.25$), respectively. The mean disease duration from symptom onset to death of the CBD-CBS and CBD-RS groups was 6.7 years and 4.3 years, respectively ($P = 0.07$). The demographic features of these nine cases are summarized in Table 1.

Case summary

Case 1 (London, UK)

This case was included in our published CBD case series (Ling *et al.*, 2010). This male had motor tics from the age of 8 and developed vocal tics in his late teens. He had lifelong anxiety disorder and an obsessive-compulsive personality trait. Four generations of his family had had a history of motor tics. He was followed up with the neuropsychiatrists at the National Hospital for Neurology and Neurosurgery, Queen Square, London, during which he joined the Queen Square Brain Bank donor programme. He died of metastatic carcinoma of the prostate at the age of 63.

Tau immunohistochemistry revealed a moderate number of astrocytic plaques in the anterior frontal region. Occasional neurofibrillary tangles, pretangles and neuropil threads were seen mainly in the anterior frontal, entorhinal and transentorhinal cortices. Sparse neuropil threads were observed in the posterior frontal, parietal and temporal regions. No ballooned neurons were found on αB-crystallin immunohistochemistry. In the striatum, there were frequent astrocytic plaques, moderate threads, few neurofibrillary tangles and occasional oligodendroglial coiled bodes. In the putamen, tau pathologies were more prominent in the medial than lateral region. Moderate neuropil threads and few neurofibrillary tangles were seen in the amygdala, substantia nigra and subthalamic nucleus. A few neuropil threads were observed in the locus coeruleus. Scattered neurofibrillary tangles and threads were observed in the midbrain tectum and tegmentum, pontine tegmentum and cerebellar dentate nucleus. Astrocytic plaques, neurofibrillary tangles, pretangles and threads were immunoreactive for 4-repeat tau but not 3-repeat tau. Mild pigment incontinence was seen in the substantia nigra. The volume of the subthalamic nucleus, locus coeruleus and cerebellar dentate nucleus was well-preserved.

Table 1 Characteristics and secondary pathologies of preclinical CBD (Cases 1–3) and end-stage CBD (Cases 4–9) cases

Case No.	Gender	Clinical diagnosis	Age at death (years)	Disease duration from symptom onset (years)	Clinically more affected side	Whole brain weight (g)	Hemisphere examined[a]	NIA-AA score (level of AD neuropathological change) (Hyman *et al.*, 2012)	TDP-43 proteinopathy	Argyrophilic grains (Saito *et al.*, 2004)	C9ORF72 inclusions[b]	α-Synuclein pathology	Vascular pathology	CAA
Case 1 (Preclinical, QSBB)	Male	Tourette's syndrome	63	NA	NA	1441	Left	A0, B1, C0 (Not)	-	-	-	-	-	-
Case 2 (Preclinical, New York)	Male	US Aging Project	89	NA	NA	1315	Right	A1, B0, C0 (Low)	-	-	-	-	-	-
Case 3 (Preclinical, Vienna)	Female	Kidney transplant	76	NA	NA	1120	Left	A0, B1, C0 (Not)	-	Yes	-	-	-	-
Case 4 (End-stage CBD-CBS)	Male	CBS	78	8	Right	1216	Right	A1, B1, C1 (Low)	-	-	-	-	-	-
Case 5 (End-stage CBD-CBS)	Male	CBS	72	5	Left	1378	Left	A0, B2, C0 (Not)	-	-	-	-	Mild SVD	-
Case 6 (End-stage CBD-CBS)	Male	CBS	73	7	Left	1154	Left	A0, B1, C0 (Not)	-	-	-	-	Mild SVD	-
Case 7 (End-stage CBD-RS)	Female	RS	68	5	NA	1034	Left	A1, B1, C1 (Low)	Limbic	Yes	-	-	-	-
Case 8 (End-stage CBD-RS)	Male	RS	64	4	NA	1210	Left	A1, B1, C1 (Low)	Limbic	Yes	-	-	Mild SVD	-
Case 9 (End-stage CBD-RS)	Male	RS	66	4	NA	1200	Right	A1, B1, C1 (Low)	Limbic	Yes	-	-	-	Mild

AD = Alzheimer's disease; CAA = cerebral amyloid angiopathy; NA = not applicable; NIA–AA = National Institute on Aging – Alzheimer Association; NK = not known; QSBB = Queen Square Brain Bank for Neurological Disorders, London, UK; SVD = small vessel disease; - = negative/absent.

[a]Hemisphere examined was randomly selected.

[b]Screening for inclusions using p62 immunohistochemistry in hippocampus and cerebellum.

Case 2 (New York, USA)

This male was a participant in the Washington Heights and Inwood and Columbia Aging Project and was clinically referred to as a 'healthy control' brain donor at the New York Brain Bank. His last scheduled neurological assessment, which took place a few months prior to his death at age 89, concluded 'normal cognition' and neurological examination was normal.

Tau immunohistochemistry revealed a moderate number of astrocytic plaques in the frontal cortex and striatum and, to a lesser extent, in the temporal region and rarely in the parietal region. There were also scattered neurofibrillary tangles and neuropil threads in these cortical regions as well as in the hippocampal formation, amygdala, striatum, globus pallidus, midbrain tectum, tegmentum, substantia nigra, locus coeruleus and pontine tegmentum. Rarely, a couple of scattered coiled bodies were observed in the cortical regions. These tau lesions were immunoreactive for 4-repeat tau but not for 3-repeat tau. No ballooned neurons were found on αB-crystallin immunohistochemistry. There was no evidence of cell loss in the cortices, substantia nigra, locus coeruleus, subthalamic nucleus and dentate nucleus.

Case 3 (Vienna, Austria)

The clinical and pathological features of this case were described in a case report (Milenkovic and Kovacs, 2013). In brief, this female had polycystic kidney disease and chronic renal insufficiency. Shortly after renal transplantation, she developed acute graft rejection and was treated with immunosuppressants, but eventually succumbed to septic shock and multiple organ failure at the age of 76.

Mild neuronal loss and gliosis were observed in the parietal, temporal and entorhinal cortices and substantia nigra. Moderate gliosis was observed in the caudate. Occasional ballooned neurons immunoreactive for αB-crystallin were found only in the anterior cingulate cortex. Mild-to-moderate tau pathologies including astrocytic plaques, neurofibrillary tangles, pretangles, threads and occasional coiled bodies were seen in the frontal cortex, striatum, substantia nigra, hippocampus, amygdala and, to a lesser extent, parietal, temporal and entorhinal cortices, globus pallidus and subthalamic nucleus. Few neuropil threads were detected in the locus coeruleus. Argyrophilic grains (stage II) immunoreactive for 4-repeat tau and p62 antibodies were observed in the hippocampal formation (Saito *et al.*, 2004). Mild pigment incontinence was seen in the substantia nigra. There was no evidence of neuronal loss in the frontal cortex, subthalamic nucleus and locus coeruleus.

Quantitative analysis of tau load

Regional tau load

The mean regional tau load of the preclinical CBD group was less than that of the end-stage CBD group with statistically significant difference identified in 16 selected regions ($P < 0.05$). For the remaining four selected regions, there was borderline significance in the anterior frontal grey matter, parietal white matter and putamen, and no statistical significance in the amygdala ($P = 0.15$) (Figs 2 and 3). After adjusting for multiple comparisons, significant difference in mean regional tau load between the preclinical and end-stage groups was identified in the following regions: posterior frontal grey and white matter, temporal grey matter, caudate nucleus, midbrain tectum, and pontine tegmentum. The regional tau load data of selected brain regions of the preclinical CBD cases are illustrated in Supplementary Fig. 1.

Frontal tau load distribution

Of all the cortical regions, the most abundant tau load in the preclinical CBD group was identified in the frontal cortex. Within the frontal cortex, the predominant tau load was observed in the anterior frontal region and tau lesions in the posterior frontal region were very sparse (Fig. 4). This finding was in contrast with the severe tau load in the posterior frontal region usually found in end-stage CBD-CBS cases. The mean anterior-to-posterior frontal tau load ratio (including both grey and white matter) of the preclinical CBD group was 16.04 and that of the end-stage CBD group was 1.36. Thus, the anterior-posterior gradient of tau distribution in the frontal region of the preclinical CBD group was almost 12-fold greater than that of the end-stage CBD group.

Total, cortical and basal ganglia tau load

The total ($P = 0.04$) and basal ganglia ($P = 0.001$) tau load of the preclinical CBD group were significantly less than those of the end-stage CBD group. Although cortical tau load of the preclinical CBD group was numerically less than that of the end-stage CBD group, there was no significant difference statistically ($P = 0.19$) (Supplementary Fig. 2).

Cellular lesion types

Overview

The distribution and severity of different tau lesion types based upon quantitative analysis are illustrated in Fig. 5. In the preclinical cases, an anterior-posterior gradient in the distribution of all cellular lesion types could be observed in the frontal cortex with these lesions being mainly restricted to the anterior frontal region rather than the posterior frontal region.

Neuronal lesions and neuropil threads

In end-stage CBD, neuronal lesions (neurofibrillary tangles and pretangles) were most prominent in the frontal and parietal cortices, amygdala, caudate, subthalamic nucleus and pontine tegmentum; while in preclinical CBD, neuronal lesions were mainly found, to a lesser extent than in the end-stage cases, in the anterior frontal and parietal regions, amygdala and the basal ganglia (Fig. 5). In end-stage CBD,

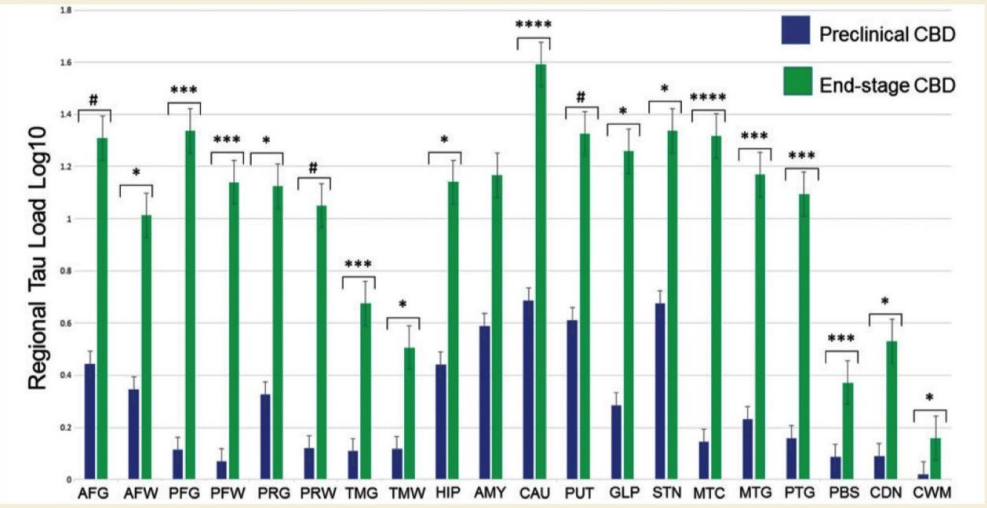

Figure 2 Mean regional tau load (Log_{10}) of preclinical CBD and end-stage CBD groups in 20 selected brain regions. Error bars represent one standard error of the mean (SEM). $^{****}P < 0.001$, ****P*-value: 0.001–0.005, ***P*-value: 0.005–0.01, **P*-value: 0.01–0.05, $^{\#}$*P*-value: 0.05–0.01 (borderline statistical significance), Student's *t*-test. AFG = anterior frontal grey matter; AFW = anterior frontal white matter; AMG = amygdala; CAU = caudate; CDN = cerebellar dentate nucleus; CWM = cerebellar white matter.; GLP = globus pallidus; HIP = hippocampus; MTC = midbrain tectum; MTG = midbrain tegmentum; PBS = pontine base; PFG = posterior frontal grey matter; PFW = posterior frontal white matter; PRG = parietal grey matter; PRW = parietal white matter; PTG = pontine tegmentum; PUT = putamen; STN = subthalamic nucleus; TMG = temporal grey matter; TMW = temporal white matter.

severe neuropil thread pathology was observed in the frontal region, basal ganglia, midbrain tectum, whereas in the preclinical cases, mild thread pathology was found in the frontal cortex, amygdala and basal ganglia and was almost absent in other regions with scattered neuropil threads occasionally observed in the temporal and parietal cortices and cerebellar white matter.

Astrocytic plaques

In end-stage CBD cases, moderate numbers of astrocytic plaques were observed in the frontal, parietal and temporal cortices, amygdala and striatum. In the preclinical cases, astrocytic plaques were most abundant in the striatum, and were observed in mild-to-moderate density in the anterior frontal, parietal and temporal cortices. Astrocytic plaques were uncommon in the brainstem and cerebellum in both groups.

Coiled bodies

In our end-stage CBD cases, oligodendroglial coiled bodies were in general less common than other lesion types and were occasionally observed in the cortical white matter, lentiform nucleus and brainstem, whereas in preclinical CBD, very occasional coiled bodies were observed in the anterior frontal region and lentiform nucleus.

Tau lesion types in the cortical regions

In the end-stage CBD cases, neuronal lesions in the cortical regions were at least four times more abundant than astrocytic plaques; while the proportion of these two lesion types was similar in the preclinical CBD cases (Supplementary Fig. 3). The average ratio of neuronal lesions to astrocytic plaques in the four cortical regions (anterior and posterior frontal, parietal and temporal cortical grey matter) was 0.91 in the preclinical CBD group and 4.20 in the end-stage CBD group ($P < 0.001$; χ^2 test).

Multiple regression analysis was performed to investigate which of the cellular lesion type best correlates with regional tau load in cortical grey matter (Supplementary Fig. 4). In the preclinical CBD cases, there was a significant correlation between neuronal lesions and regional tau load ($P = 0.002$, $R^2 = 0.98$), while in the end-stage CBD cases, neuropil threads ($P < 0.001$) and neuronal lesions ($P = 0.056$, borderline significance) significantly correlated with regional tau load ($R^2 = 0.87$). Other lesion types such as astrocytic plaques and coiled bodies did not correlate with the regional tau load in either CBD group.

Tau lesion types in the hippocampal formation

The distribution and severity of neuronal and thread pathologies in the hippocampal formation are illustrated

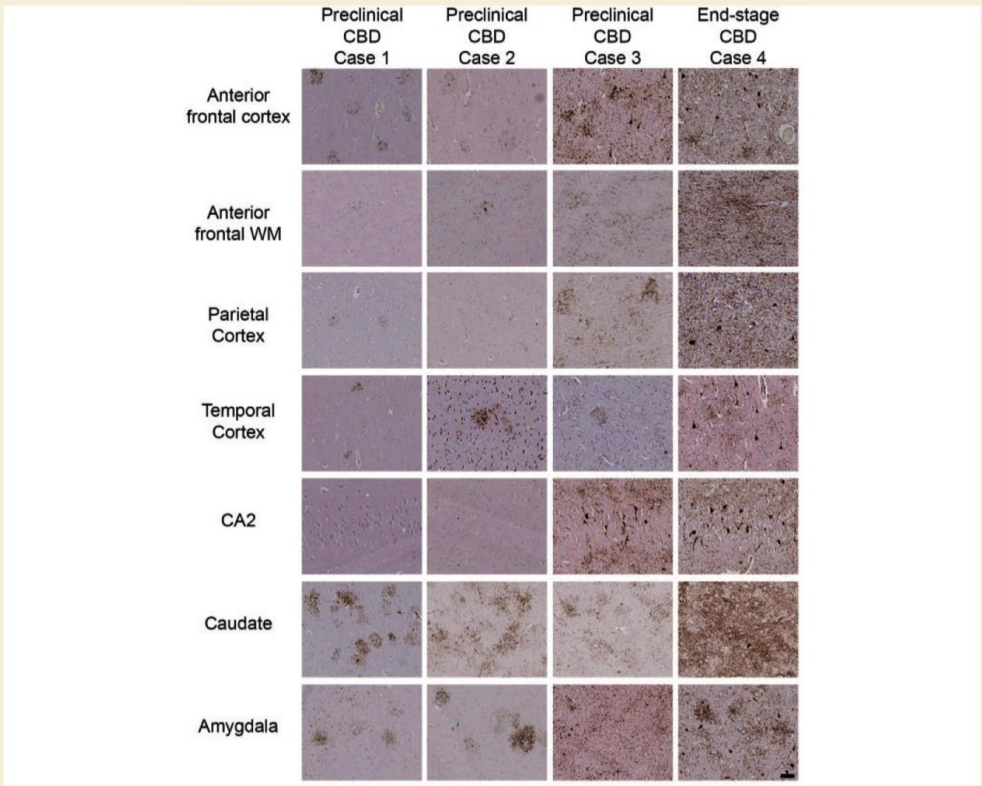

Figure 3 Tau immunohistochemistry (AT8) sections of the three preclinical CBD cases (Cases 1–3) and one end-stage CBD-CBS case (Case 4). Scale bar = 38.5 μm. WM = white matter.

in Supplementary Fig. 5. Tau lesions in the hippocampal subregions were very mild in preclinical Cases 1 and 2. In preclinical Case 3 and most of the end-stage CBD cases, severe neuronal and thread pathologies were observed in CA1 and the granular cell layer even in cases with no or minimal Alzheimer's-related changes and absence of argyrophilic grains (e.g. Cases 4–6). In preclinical CBD cases, mild astrocytic plaques were observed in CA1, subiculum (Cases 1 and 2), and entorhinal cortex (Cases 1 and 3). Astrocytic plaques were observed in the entorhinal cortex of three of the six end-stage CBD cases, ranging from mild (Cases 4 and 8) to severe (Case 9).

AT100 immunohistochemistry

For both the preclinical and end-stage CBD cases, the tau-positive lesions (neurofibrillary tangles, pretangles, neuropil threads, astrocytic plaques and coiled bodies) were also immunoreactive with antibody AT100 (Fig. 1). This finding indicated that disease-associated tau in all lesion types observed in the preclinical cases was phosphorylated at serine-212 and threonine-214 and was in advanced stage of the aggregation process and formed filaments i.e. tau was in the same conformation as the tau lesions in end-stage cases (Clavaguera *et al.*, 2009).

Cell loss in substantia nigra

Semiquantitative assessment of cell loss using a four-tier rating scale in five substantia nigra subregions showed that cell loss was either absent or very mild in the preclinical cases, while in the end-stage cases, the nigral cell loss ranged from moderate to severe (medial, dorsomedial and ventromedial subregions: $P = 0.03$, χ^2 test), with the ventrolateral subregion being most severely and consistently affected in all six end-stage cases ($P = 0.01$; χ^2 test); one exception was in the dorsolateral subregion ($P = 0.06$,

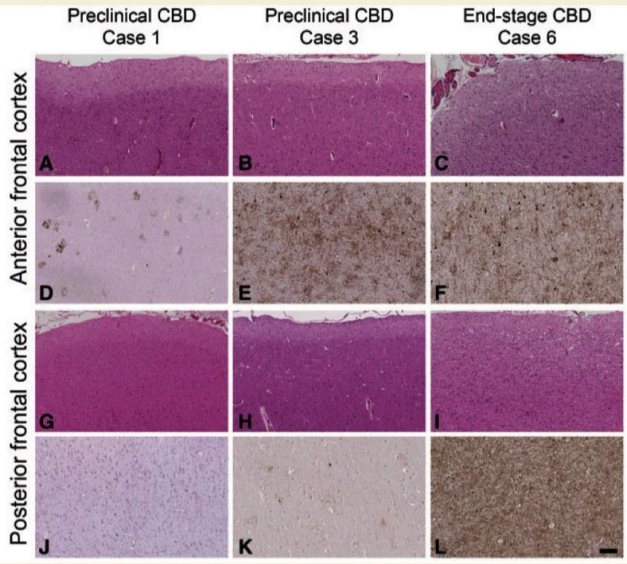

Figure 4 Anterior and posterior frontal cortex of two preclinical CBD (Cases 1 and 3) and one end-stage CBD-CBS case (Case 6). The characteristic features of cortical neuronal loss and superficial spongiosis are observed in the end-stage CBD case (**C** and **I**) but not in the preclinical CBD cases (**A**, **B**, **G** and **H**). In the preclinical CBD cases, tau immunohistochemistry (AT8) demonstrates tau lesions, mainly in the form of astrocytic plaques, which are more abundant in the anterior frontal cortex (**D** and **E**) than in the posterior frontal cortex (**J** and **K**). This anterior-posterior gradient is most apparent in Case 1 (**D** and **J**), which has relatively less total tau load and is thought to represent an earlier stage of CBD pathology than Case 3. On the contrary, in the end-stage CBD case, neurofibrillary tangles and neuropil threads are much more frequent than astrocytic plaques and the tau distribution is more severe in the posterior frontal cortex (**L**) than in the anterior frontal cortex (**F**). Haematoxylin and eosin: **A–C**, **G–I**; tau immunohistochemistry (AT8): **D–F**, **J–L**. Scale bar = 100 μm.

borderline significance; χ^2 test) where cell loss was found to be mild in a CBD-CBS case (Case 4) (Fig. 6).

Secondary pathologies

Secondary pathologies in preclinical and end-stage cases are summarized in Table 1. Argyrophilic grains were observed in one preclinical case (Case 3, Vienna). Mild cortical amyloid-β pathology was observed in another preclinical case (Thal phase 1, Case 2, New York, age of death: 89 years). TDP-43, α-synuclein, vascular and cerebral amyloid angiopathy pathologies were not identified in any of the preclinical cases.

MAPT gene sequencing

Sanger sequencing of *MAPT* gene for any known pathological mutations in exons 10–13 were negative in all three preclinical CBD cases. Preclinical Cases 1 and 3 had H1/H1 and Case 2 had H1/H2 *MAPT* haplotypes.

Discussion

We described the histological features of three clinically asymptomatic cases with CBD-tau lesions in both neurons and glia. These lesions were immunoreactive with the AT8, AT100 and anti-4-repeat tau antibodies but not with the antibody to 3-repeat tau. Astrocytic plaques with Gallyas-positive and tau-imunoreactive annular clusters of short processes were seen, most prominently in the striatum followed by the anterior frontal and parietal regions. The severity of neuronal lesions and neuropil threads followed a rostro-caudal descending gradient from the forebrain to the hindbrain structures but the overall tau burden in the preclinical cases was significantly less than the end-stage CBD cases. Cortical neuronal loss, spongiosis, ballooned neurons, thinning of the corpus callosum, nigral cell loss, all of which are considered to be characteristic features of end-stage CBD, were consistently observed in all six of our end-stage CBD cases, but were either absent or minimal in the three preclinical cases. We interpreted these histological findings as early CBD pathology and the lack of clinical symptoms was due to subthreshold pathology.

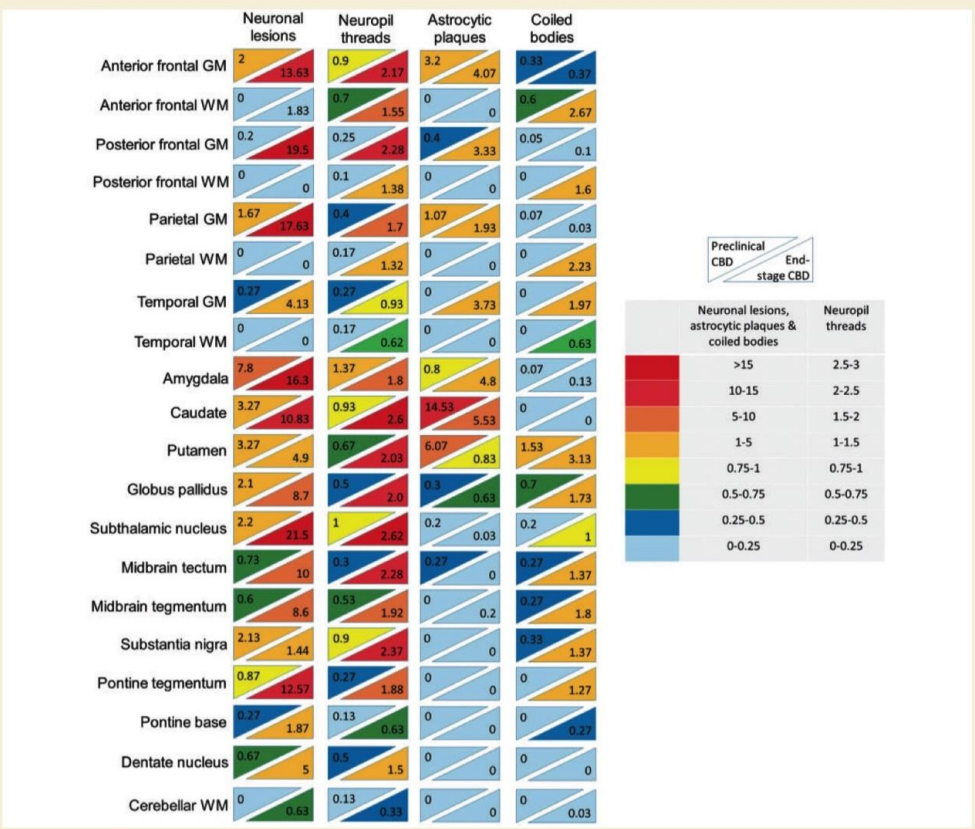

Figure 5 Distribution and severity of tau-immunoreactive cellular lesions in preclinical and end-stage CBD groups. A summary of the tau pathology in three preclinical CBD and six end-stage CBD (three CBD-CBS and three CBD-RS) cases using mean lesion count for neuronal lesions, astrocytic plaques and coiled bodies and a four-tier semiquantitative scores for neuropil thread (0 = none, 1 = mild, 2 = moderate, 3 = severe) assessed at 20× objective in five random fields (except for substantia nigra, subthalamic and dentate nuclei where three random fields were used due to small regional areas). The severity of tau pathology is colour-coded with a heat map, with more severely affected areas showing hotter colours (red, orange and yellow) and less affected areas represented by cooler colours (green and blue). GM = grey matter; WM = white matter.

The distribution and severity of neuronal loss and tau pathology are closely associated with the clinical syndrome in CBD as in other neurodegenerative conditions (Boxer *et al.*, 2006; Whitwell *et al.*, 2010; Ling *et al.*, 2013). The lack of significant neuronal loss in the cortex, substantia nigra and less total tau load in our preclinical CBD cases when compared with end-stage CBD cases suggest that a threshold of pathological burden in the 'right' anatomical regions needs to be reached for the onset of clinical symptoms. Despite the relatively smaller number of tau lesions, the tau pathology in preclinical cases is widespread and shows a topographical distribution that is overall similar to that observed in end-stage cases. As the total tau load of Case 3 was 4- and 5-fold greater than Cases 1 and 2, it is reasonable to assume that Cases 1 and 2 are at an earlier stage of the pathological process than Case 3.

In Case 1, cortical pathology was very mild and was mainly observed in the anterior frontal region, followed by the posterior frontal region, relatively dense tau pathology was also found in the striatum and subthalamic nucleus. In Case 2, tau pathology was similarly distributed in the frontal and temporal grey and white matter. In Case 3, predominant cortical tau pathology was observed in the anterior frontal and parietal regions as well as in the

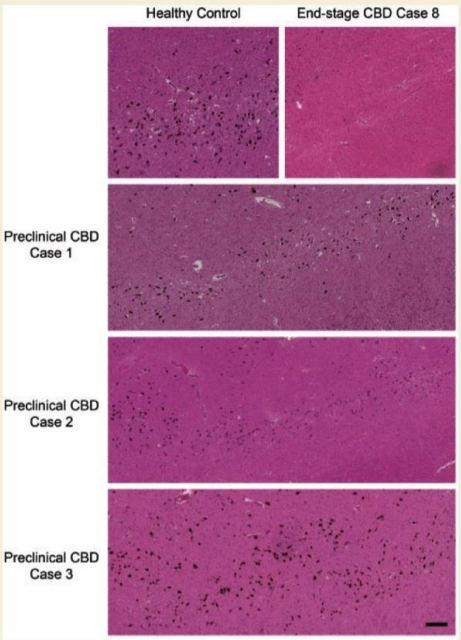

Figure 6 Haematoxylin and eosin sections of the substantia nigra. The substantia nigra of the three preclinical CBD Cases 1–3 and an 82-year-old healthy control are relatively preserved with a good population of pigmented neurons. Severe loss of pigmented neurons, gliosis and scaring are observed in the substantia nigra of the end-stage CBD-RS Case 8. Scale bar = 100 μm.

hippocampus, amygdala and caudate with moderate gliosis in the caudate. In a quantitative study comparing the tau distribution of CBD-CBS and CBD-RS, Kouri *et al.* (2011) showed that CBD-CBS cases had significantly greater tau burden in the primary motor and somatosensory cortices of both grey matter and white matter and putamen than in CBD-RS cases and that tau burden in the anterior frontal cortex (superior frontal gyrus) was the same in both CBD phenotypes. Phenotypic presentation of CBD is probably predetermined at a very early stage as seen in our preclinical cases. If these individuals had lived for longer and the disease process was allowed to evolve, Case 2 may have developed an FTD syndrome based on the early predominant tau distribution in the frontal and temporal regions, while Cases 1 and 3 may have had the classic CBS phenotype in view of the early frontal and striatal involvement. Similar to most brain bank protocols, only one brain hemisphere of each case was processed for histological studies and was available for this study. In view of the predilection of tau accumulation in different cortical regions early in the disease process, it is possible that interhemispheric asymmetry of pathology is already evident in these preclinical cases (Oide *et al.*, 2002; Boxer *et al.*, 2006; Hassan *et al.*, 2010), but remains at a subthreshold level bilaterally.

In Cases 1 and 3, tau pathology was more prominent in the anterior frontal region (prefrontal cortex) than in the posterior frontal (precentral gyrus involving primary motor cortex). Unfortunately, the posterior frontal region in Case 2 was not available for analysis. A 12-fold greater anterior-to-posterior frontal tau load ratio was found in these preclinical cases when compared with the end-stage group. This anterior-posterior gradient suggests the initial site of tau accumulation in the frontal cortex is likely to be the anterior frontal region, while the posterior frontal area is involved at a later stage. Symptom onset of CBS probably coincides with the threshold of pathological burden in the primary motor cortex being reached.

The distribution of cellular lesion types differed between the preclinical and end-stage cases. In the anterior frontal cortex, the mean astrocytic plaque count was 2.5 (range: 0–10; averaging over five random fields at 20× objective) in Cases 1 and 2 with sparse threads and absence of neuronal lesions, while the mean neuronal and astrocytic plaque counts in the anterior frontal cortex of Case 3 were 6 and 4.6, respectively. In end-stage cases, neuronal lesions in the anterior frontal cortex were three times greater than astrocytic plaques (13.6 to 4.1). Likewise, in the striatum, a region with one of the highest mean tau loads in both CBD groups, the mean neuronal and astrocytic plaque counts in Cases 1 and 2 were 2.2 and 13.0, and in Case 3, they were 5.4 and 5, respectively. In the end-stage CBD cases, neuronal lesions in the striatum were twice as many as astrocytic plaques (7.9 to 3.2). These findings indicate that astrocytic plaques were the predominant lesion type in the anterior frontal cortex and striatum of Cases 1 and 2, which were thought to be at an earlier disease stage than Case 3. It is possible that astrocytic plaques are the earliest tau lesion type that occurs in regions affected early by the CBD pathological process, leading us to speculate that CBD may begin as an astrogliopathy. Based upon the findings in Case 3 and end-stage cases, it appears that as the pathology progresses, neuronal lesions markedly increase in number and eventually overtake astrocytic plaques as the predominant lesion type. Notably, in some end-stage cases, very dense thread pathology observed in the cerebral cortex and some subcortical grey nuclei may mask a proportion of the astrocytic plaques. Josephs *et al.* (2006) studied the correlation between disease duration and lesion types in PSP and noted that cases with the most severe neuronal tau pathology had the longest disease duration, indicating that neuronal tau pathology, rather than astrogliopathy, is the most abundant cellular lesion type in end-stage PSP, another 4-repeat tauopathy. Multiple regression analysis has identified a correlation between neuronal lesions and regional tau load in the preclinical group. This finding may be explained by the larger tau-immmunoreactive area attributed by neuronal lesions in Case 3.

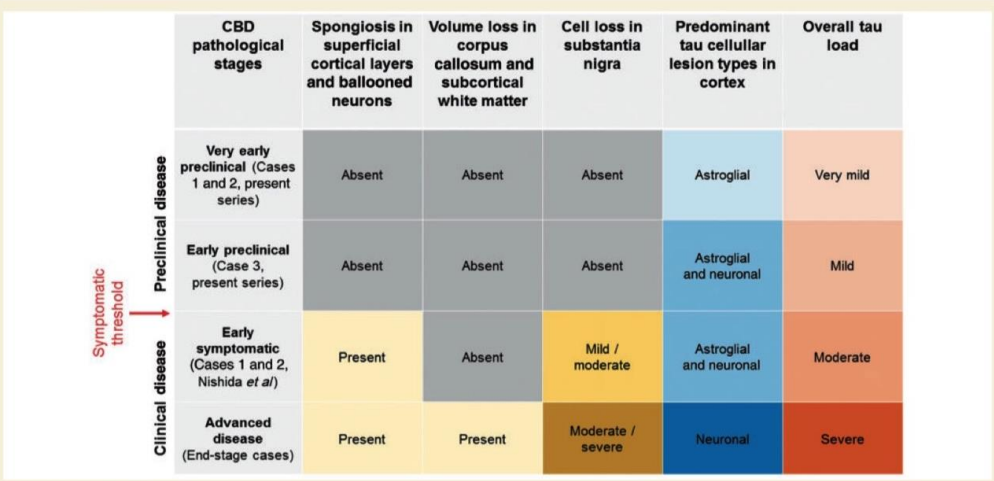

Figure 7 Characteristic features of the pathological progression of CBD.

In end-stage CBD, there is a variable degree of neuronal loss and gliosis in the substantia nigra (Dickson *et al.*, 2002). Neuronal loss in the ventrolateral nigral cell groups correlates with extrapyramidal features in parkinsonism (Fearnley and Lees, 1991); whereas neuronal loss in the medial nigra correlates more with cognitive and frontal behavioural deficits (Rinne *et al.*, 1989) and was more severely affected in CBD-RS than CBD-CBS (Kouri *et al.*, 2011). This finding was also confirmed by the present study. Our end-stage CBD-RS cases had more severe neuronal loss in the medial ($P = 0.014$) and dorsomedial ($P = 0.014$) tiers when compared with CBD-CBS cases and severe neuronal loss (grade 3 all) was observed in the ventrolateral tier in both phenotypes. Pirker *et al.* (2015) reported two post-mortem confirmed CBD cases with mildly reduced tracer uptake in dopamine transporter single-photon emission computed tomography (SPECT) scan in early disease stage (1.5 years after symptom onset), and subsequently, the tracer uptake markedly declined later in the disease course (4.5 and 5 years after symptom onset). Our group previously reported a pathologically confirmed CBD case with normal dopamine transporter SPECT tracer uptake more than 4 years into the illness (O'Sullivan *et al.*, 2008). These findings suggest that nigrostriatal degeneration may be a late pathological feature of CBD. In our preclinical cases, tau pathology in the substantia nigra was mild and the cell population in all nigral subregions was either preserved or showed very mild cell loss (grade 0 to 0.5 in all subregions of all three preclinical cases), indicating nigral cell loss takes place later in the disease course.

Severe neuronal loss and gliosis in the subthalamic nucleus is a prerequisite for the pathological diagnosis of PSP (Hauw *et al.*, 1994), but it is not a consistent feature in end-stage CBD. Only three of the six end-stage CBD cases showed mild neuronal loss with gliosis in the subthalamic nucleus, while the volume of the remaining three cases was preserved. In the preclinical cases, the subthalamic nucleus has one of the highest tau burdens and tau lesions (mild-to-moderate neurofibrillary tangles, pretangles and neuropil threads), with preserved volume. Neuronal loss of the subthalamic nucleus is a late feature in the CBD disease process, which is likely related to the downstream pathological involvement from the striatum, pars externa of the globus pallidus and substantia nigra within the basal ganglia circuit.

In preclinical Cases 1 and 2, very mild tau lesions were observed in the hippocampal formation. There were more severe neuronal lesions in preclinical Case 3 but they may be attributable to co-existing argyrophilic grain disease pathology. In the fimbria, only very mild thread pathology was observed in Cases 1 and 2 but this was of moderate degree in Case 3. These findings suggest that tau pathology is minimal in the hippocampal formation in very early disease stage. It is likely that as the disease progresses, the hippocampus and fimbria, which is the white matter outflow of the hippocampus forming the fornix, become gradually affected by tau pathology. Similar findings were observed in the amygdala, of which the regional tau load was very mild in Cases 1 and 2 (regional tau load: Case 1 = 0.51, Case 2 = 0.48) and was much greater in Case 3 where argyrophilic grains were also present (regional tau load = 24.97) (Supplementary Fig. 1). Based on the findings in Cases 1 and 2, tau pathology in the amygdala is probably mild in very early disease stages in the absence of secondary pathology. Mild tau pathology in the amygdala

was also observed in an incidental case (Case 1) recently reported by Nishida *et al.* (2015).

Only four preclinical CBD cases have been reported in the literature, two of which are included in this series [Cases 1 (Ling *et al.*, 2010) and 3 (Milenkovic and Kovacs, 2013)]. Recently, two preclinical (or early symptomatic) cases, along with one clinically symptomatic end-stage CBD-bvFTD case, were identified from 887 brains in a forensic autopsy series in Toyama, Japan, over a 6-year period, giving a pathological incidence rate of 0.34% (Nishida *et al.*, 2015). Case 1 of the Japanese report was a 65-year-old male who had died of smoke inhalation at the scene of a house fire. Case 2 was a 77-year-old female diagnosed with dementia 1 month prior to death with a Mini-Mental State Examination score of 21/30, but did not experience any speech or motor impairments. She died of accidental drowning. Both cases had subtle clinical features consistent with early cognitive impairment. Histological examination of both cases revealed widespread tau pathology including neurofibrillary tangles, pretangles, astrocytic plaques in the fontal, parietal and temporal cortices, limbic, basal ganglia and brainstem structures. Ballooned neurons were observed in the frontal, temporal and limbic cortices. There was moderate neuronal loss in the substantia nigra in both cases. Numerous argyrophilic grains were found in the amygdala in Case 2 (stage II) (Saito *et al.*, 2004; Nishida *et al.*, 2015). The authors concluded that milder overall tau burden, sparse tau pathology in the superficial cortical layers, and the lack of volume loss or gliosis in the cortices, subcortical white matter and the corpus callosum as shown in their two cases, were likely to be early histological features of CBD. In addition to these characteristics, our preclinical cases also exhibited much milder thread pathology, which was restricted to the anterior frontal white matter and absent in other subcortical white matter, absent or rare ballooned neurons and preserved substantia nigra, suggesting our cases may represent an even earlier stage of CBD pathology when compared to the two cases described by Nishida *et al.* (2015).

While the preclinical cases in the present series represent very early (Cases 1 and 2) and early (Case 3) CBD pathology, the Japanese cases (Cases 1 and 2) (Nishida *et al.*, 2015) most likely illustrate a continuum of early symptomatic CBD pathology (Fig. 7). A pathological threshold that coincides with clinical disease onset is probably marked by an overall increase in tau burden, especially in strategic brain regions accompanied by cortical spongiosis, nerve cell loss and the appearance of ballooned neurons as well as nigral neuronal loss (Fig. 7). Future post-mortem studies of symptomatic patients with CBD who have died of other causes prior to reaching end-stage disease, will be of great value to demonstrate intermediate pathology prior to reaching a fully advanced disease stage.

Argyrophilic grains can be observed in over 40% of end-stage CBD cases (Togo *et al.*, 2002). The findings of argyrophilic grains in one of our preclinical cases (Case 3, Vienna) and in another early CBD case (Case 2) in the Japanese series suggest that argyrophilic grains are a concomitant pathological feature that occurs early in the CBD pathological process. It is possible that these two distinct 4-repeat tauopathies, CBD and argyrophilic grain disease, share common characteristics that are relevant for the pathogenesis of both diseases. Moreover, the occasional ballooned neurons observed in limbic structures in our Case 3 are most likely driven by the co-existing argyrophilic grain disease rather than by the CBD pathological process (Saito *et al.*, 2004).

Preclinical cases of Alzheimer's disease and Lewy body disease are common and their prevalence is much greater than the prevalence of their symptomatic counterparts (Bouras *et al.*, 1994; Beach *et al.*, 2009; Adler *et al.*, 2010). On the other hand, preclinical cases of other neurodegenerative conditions are very rare, only six preclinical PSP (Oshima *et al.*, 2004; Evidente *et al.*, 2011) and two preclinical multiple system atrophy cases (Parkkinen *et al.*, 2007; Fujishiro *et al.*, 2008) have been described in the literature. Evidente *et al.* (2011) reported five preclinical PSP cases with frequent pathognomonic tufted astrocytes in the characteristic brain regions. An association between H1 haplotype and 4-repeat tauopathies, including PSP and CBD, was previously reported and H1/H1 haplotype was identified in our preclinical Cases 1 and 3 (Houlden *et al.*, 2001).

Our Case 1 died at the age of 63, but notably, Case 2 in the present series died at age 89 and our Case 3 and Case 2 of the Japanese series also died at a relatively old age of 76 and 77, respectively. Assuming that all five preclinical cases reported in the present and Japanese series had lived longer and that all cases went on to develop clinical symptoms, their estimated age at symptom onset (mean = 74) would have been significantly higher than that of the six end-stage CBD cases used as controls in the present study (mean = 64.2, $P = 0.07$, borderline significance) and that of our large end-stage CBD cohort ($n = 109$; mean = 64.6 years, $P = 0.01$). In view of the fact that three of the five available preclinical cases were significantly older, there remains a possibility that some of these cases may represent a heterogeneous subgroup distinct from the typical CBD cases and our findings should be interpreted with caution. Future studies are required to explore whether some of these older preclinical cases represent a more 'benign' subgroup due to the presence of distinct protective factors (such as H2 allele in our Case 2) or cognitive reserve (Case 2 also had a demanding professional occupation). This notion would be supported by existing findings in other neurodegenerative diseases such as Parkinson's disease and PSP, of which phenotypic subgroups are segregated by age and disease duration (Williams *et al.*, 2005; Halliday *et al.*, 2008).

The study of preclinical CBD cases provides valuable insights into the early regions and lesion type affected by the CBD pathological process. These findings may serve as a basis for *in vivo* tau-imaging studies in patients with early symptoms to predict underlying CBD pathology, and the

development of disease-modifying treatments targeting early disease. Although the rarity of these preclinical cases coming to post-mortem makes further clarification of the true nature of these preclinical cases unlikely unless clinicopathological studies with a very large sample size are conducted. The widespread neuronal and glial tau lesions found in a typical anatomical distribution as observed in end-stage CBD and the presence of pathognomonic astrocytic plaques support our view that these cases represent early CBD and would have eventually evolved to symptomatic CBD.

In summary, the earliest pathological features of CBD deduced from these three preclinical cases are: (i) overall less tau burden; (ii) astrocytic plaques are an early lesion type most prominent in the prefrontal cortex and striatum; (iii) relatively higher tau load in the anterior frontal cortex, striatum and subthalamic nucleus, suggesting striatal afferent connection to the dorsolateral prefrontal cortex and the basal ganglia circuitry may be the earliest neural network connections affected by CBD-tau (Ahmed *et al.*, 2016); (iv) preserved substantia nigra; and (v) either absent or minimal cortical neuronal loss, superficial spongiosis and ballooned neurons.

Note

Since the submission of this manuscript, another preclinical CBD case report of a 93-year-old man with mixed pathologies, including early CBD features, has been published (Martinez-Maldonado *et al.*, 2016). The absence of neuronal loss and spongiosis in the superficial cortical layer, sparse ballooned neurons in the cortex and sparse thread pathology reported in this latest preclinical case are consistent with our findings.

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Conflicts of interest

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Other authors report no conflict of interest.

Supplementary material

Supplementary material is available at *Brain* online.

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